Identification and Characterization of the Ion Channel TRPM8 in Prostate Cancer

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Zusammenfassung

Das Prostatakarzinom ist die häufigste Krebserkrankung des Mannes. Bei den zu Tode führenden Tumoren wird es im Jahre 2003 nach dem Bronchialkarzinom an 2. Stelle stehen. Diese Inzidenz zeigt, dass dringend neue diagnostische Marker und therapeutische Zielgene zur Behandlung von Prostatakrebs benötigt werden.

Ziel dieser Dissertation war es, mit Hilfe der DNA-Chiptechnologie neue tumorrelevante Gene für eine Small-Molecule- und Antikörper-Basierte Therapie des Prostatakarzinoms zu identifizieren. Auf einen proprietären Tumor-Chip der Firma metaGen Pharmaceuticals GmbH wurde mikrodissektiertes Normal- und korrespondierendes Tumorgewebe von 52 Prostatatumorpatienten hybridisiert. Mit Hilfe bioinformatischer Analysen der Chipergebnisse konnte das Gen TRPM8 identifiziert werden, das in Prostatatumoren in mehr als 56% der Patienten überexprimiert ist.

Northern-Blot, Dot-Blot und Chipexperimente zeigten, dass TRPM8 ungewöhnlich gewebespezifisch exprimiert wird. In mehr als 400 getesteten Tumorpatienten und in 23 Normalgeweben wurde TRPM8 ausschließlich in der Prostata und neuroendokrinen Tumoren nachgewiesen.

TRPM8 gehört zur Familie der Transient Receptor Potential Channel Proteins. Es konnte hier erstmals in Fluoreszenz-Resonanz-Energie-Transfer- Experimenten (FRET) gezeigt werden, dass TRPM8 Multi-Homomere bildet. Dies wurde bisher nur für Kanäle anderer TRP-Subfamilien (TRPV und TRPC) gezeigt.

Weiterhin konnten erstmals mehrere Spleißvarianten von TRPM8 identifiziert werden. Quantitative RT-PCR Experimente zeigten, dass diese noch stärker in Prostatatumoren überexprimiert sind als TRPM8 selbst. Des Weiteren wurde ein neues Gen auf dem DNA-Gegenstrang von TRPM8 entdeckt, das mit Exon 11 von TRPM8 100% komplementär ist und an der Regulation von TRPM8 beteiligt sein könnte.

Der Promotor von TRPM8 wurde durch eine *in silico* Analyse identifiziert und *in vitro* bestätigt. Obwohl eine starke androgenabhängige Expression von TRPM8 in LNCaP Zellen gezeigt werden konnte, wurden keine Bindungsstellen für androgenabhänginge Elemente gefunden. Allerdings ließen sich drei Bindungsstellen des androgenregulierten Homeoboxgens NKX3.1 identifizieren.

Die Ergebnisse dieser Arbeit zeigen, dass TRPM8 und seine Isoformen aufgrund ihrer Gewebspezifität ausgezeichnete Angriffspunkte für eine zielgerichtete Prostatakrebstherapie sind.

TRPM8
Prostata
Krebs
Chip
Ionenkanal

Abstract

Prostate cancer is the most commonly diagnosed malignancy in men in the Western World. In 2003 malignancies of the prostate will be the second most common fatal cancer in men after lung cancer as estimated by the American Cancer Society. Despite the tremendous efforts made in the past to improve the treatment of prostate cancer patients, there is still an urgent need for new markers and therapeutic targets for medication.

The aim of this thesis was the identification of new genes relevant in prostate cancer, which could be used in a small-molecule or antibody based therapy of prostate cancers. Microdissected matched prostate cancer and normal tissues of 52 prostate cancer patients were hybridized to a proprietary high density Cancer-Chip based on Affymetrix GeneChip technology. Using a bioinformatic analysis, it was possible to identify TRPM8, which was highly overexpressed in 56% of prostate cancer patients. Northern blot, dot blot and gene chip experiments revealed that TRPM8 expression is extremely tissue specific. Of 400 patients and 23 tissues tested, TRPM8 expression could only be detected in the prostate and neuroendocrine tumors.

Functionally, the protein belongs to the transient receptor potential channel family of non-voltage gated proteins. It could be shown for the fist time that TRPM8 subunits form homomers using FRET technology.

Molecular characterization of TRPM8 transcription revealed multiple splice forms of TRPM8. Further, it was possible to identify a new mRNA present on the opposite strand of TRPM8, which was 100% complementary to exon 11 of TRPM8, thus it could possibly function as a regulatory RNA of TRP channel. All of these isoforms were found to be even higher overexpressed in prostate tumors than TRPM8 itself.

The promoter region of TRPM8 was identified using *in silico* methods and confirmed in promoter reporter assays. Although a high androgen dependent transcriptional activation of TRPM8 could be found by RT-PCR in LNCaP cells, no androgen responsive elements was identifiable within the promoter region. On the other hand three binding sites for the androgen dependent homeobox gene NKX3.1 and several other homeobox genes were discovered.

The results of the thesis show that TRPM8 and its isoforms are, due to their tissue specificity, ideal targets for the development of new therapeutic drugs for the treatment of prostate cancer.

TRPM8
prostate
cancer
microarray
Ionchannel

Abbreviations

	A
ACCP	acidic phosphatase prostate
AR	androgen receptor
ARE element	androgen responsive
aRNA	amplified RNA
ARNT	aryl hydrocarbon receptor nuclear translocator
ATCC	American Type Culture Collection
	B
BAC	bacteria artificial chromosomes
BLAST	basic local alignment search tool
BPH	benign prostate hyperplasia
	C
$[Ca^{2+}]_i$	cytosolic Ca^{2+} concentration
cRNA	copy RNA
CCD	cooled chargecoupled device
CCE	capacitive calcium entry
CCS	charcoal (treated) serum
CFP	cyan fluorescent protein
CGH	comparative genomic hybridization
CHO	chinese hamster ovarian cells
	D
DABCO	1.4-diazobicyclo-(2.2.2)-octane
DAG	Diacylglycerol
DAPI	4'.6-diamidino-2-phenylindole
d NTPs	2'-deoxynucleotide-5'-triphosphate
DRE	Digital Rectal Examination
DRG	Dorsal root ganglia
	D
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

	E
ER	endoplasmic reticulum
EST	expressed sequence tag
Ev	empty vector
	F
F	Fluorescence
FACS	fluorescence activated cells sorter
FBS	fetal bovine serum
FISH	fluorecence in situ hybridzation
FITC	fuoresceine Isothiocyanate
FLIPR	fluorescent Imaging Plate Reader
Fp	Flavoprotein
FRET	fluorescence resonance energy transport
	G
GG	Gleason Grading
GPCR	G-protein coupled receptor
GTC	guanidine thiocyanate
	H
H	Hour
HGNC	HUGO Gene Nomenclature Committee
HRP	horse radish peroxidase
Hs	homo sapiens
HTGS	high throughput genome sequences
HUGO	human genome organisation
	K
kD	kilo Dalton
	M
MB	mega bases
Mm	mus musculus
MYC	v-myc myelocytomatosis viral oncogene homolog
	N
NE	Neuroendocrine
NFE2L1	nuclear factor (erythroid-derived 2)-like
NKX3-1	NK3 transcription factor related, locus 1 (Drosophila)

	O
ORF	open reading frame
	P
PAP	prostatic acid phosphatase
PBS	phosphate buffered saline
PE	Phycoerithrin
PIN	prostatic intraepithelial neoplasia
PLC	Phospholipase C
PMQ	perfect match quintile
PRX2	paired related homeobox protein 2
	R
Rn	rattus norvegicus
RP	radical prostatectomy
RT	reverse transcription
	S
SDHA	succinate dehydrogenase complex, subunit A,
SKY	spectral karyotyping
SOC	store operated Ca^{2+} channels
SR	serine/arginine-rich
STS	sequence-tagged site
SV	Splice variant
	T
TM	Transmembrane
TNM	tumor node metastasis
TRP	transient receptor potential
TRUS	transrectal ultrasonography
	U
UTR	untranslated region
	W
WT	wild type
	Y
YFP protein	yellow fluorescent

1 INTRODUCTION

1.1 INCIDENCE OF PROSTATE CANCER

Cancer deaths account for 23% of all deaths in Western Europe and the United States ranking second only to deaths from heart disease. When deaths are categorized by age, sex, and cause, cancer is by far the main cause of deaths among men and women between 40 and 79 years of age [Jemal, 03]. Among cancers, malignancies of the prostate are the most commonly diagnosed tumors in European and American males [Howe, 01; Kieschke, 02]. In 2003 malignancies of the prostate will be the second most common fatal cancer in men (10%) after lung cancer (31%), followed by colorectal cancer (10%) [Jemal, 03], as estimated by the American Cancer Society.

In Germany approximately 700,000 men suffer from prostate cancer. It is the most frequently (18,7%) diagnosed tumor with an incidence of 31.500 cases displacing 1998 lung cancer as the most frequently diagnosed carcinoma [Kieschke, 02]. Prostate cancer afflicts men at an average age of 72 years, 6 years above that of cancer in general. The five-year relative survival rate of prostate cancer in Germany is 70%. In general, the number of diagnosed prostatic malignancies has increased, but the numbers of deaths per year have decreased in the last years. The reasons for this increase in incidence is the aging population and most importantly, the improved early detection methods such as serum testing of prostate specific antigen (PSA) and the digital rectal examination (DRE). Early detection as well as improved surgical intervention and radiation therapy have reduced the number of deaths significantly. But prostate cancer still ranks third of all lethal cancers causing about 18,000 deaths per year in Germany [Kieschke, 02].

Although more men die with prostate cancer than of prostate cancer, there is still no effective cure for many patients suffering especially from aggressive and advanced forms of prostate cancers. PSA screening is one of very few preoperative parameters of prognostic relevance. So far it is not possible to distinguish between aggressive and minor severe forms at an early stage of the disease.

1.2 BIOLOGICAL FUNCTION OF THE PROSTATE

The prostate is a walnut-sized gland surrounding the urethra at the base of the bladder. It is surrounded by a fibroelastic capsule that penetrates the gland to divide it into lobes. The prostate contributes to the seminal fluid an alkaline liquid which is rich in spermine, phosphlipids, cholesterol, fibrinogenase, citric acid, fibrinolysin, zinc and acid phosphatase and other proteins. The seminal fluid consists further of the fluid produced in the seminal vesicles and the sperm.

The sperm, produced in the testis, enters the upper portion of the prostate through the vas deferens. Sperm and fluid from the seminal vesicles then mix with secretions emitted from the prostate to form the seminal fluid that is expelled at the time of ejaculation.

Interestingly, the prostate is neither required for viability nor for basal levels of fertility. It is widely discussed that this might be the reason for its high incidence of cancer as other vitally important organs of the urogenital system, such as the seminal vesicles and bulbourethral glands, are nearly immune to neoplasias [Abate-Shen, 00].

1.3 DEVELOPMENT OF PROSTATE CANCER

The cause of prostate cancer is still not very well understood. A distinguishing feature of this cancer is its intimate association with aging [Abate-Shen, 00]. Usually clinically detectable prostate cancer is not manifest until the age of 60 or 70. Hereditary factors account for about 10% of prostate cancers and are generally associated with an early onset of the disease [Carter, 92]. To date, two family susceptility loci have been mapped to X chromosome and to a region of chromosome 1q, although no candidate gene has been found so far [Smith, 96; Xu, 98]. Another feature is that African American men have a higher incidence and more aggressive forms of prostate cancer than white men who in turn have a higher incidence than men of Asian origin. Additionally, androgens play a povital role in all stages of the disease. High fat diets are also suspected to increase the risk of prostate cancer while a diet rich in soy may be protective. These observations have been proposed as reasons for the low incidence of this cancer in Asia [Kristal, 02].

1.4 PATHOLOGICAL CLASSIFICATIONS OF PROSTATE CANCER

Prostate cancers are generally of multifocal nature and belong the most heterogeneous tumors in humans [Macintosh, 98]. 70% of the tumors arise in the peripheral zone, whereas 15-20% arise in the central zone, and 10 -15% arise in the transitional zone.

Most of the prostate tumors are adenocarcinomas (95%), only about 4% of cases have transitional cell morphology and are thought to arise from the uroehelial lining of the prostatic urethra. Few cases have neuroendcrine morpholgy. These cells are believed to arise from the neuroendocrine stem cells normally present in the prostate (Fig. 1).

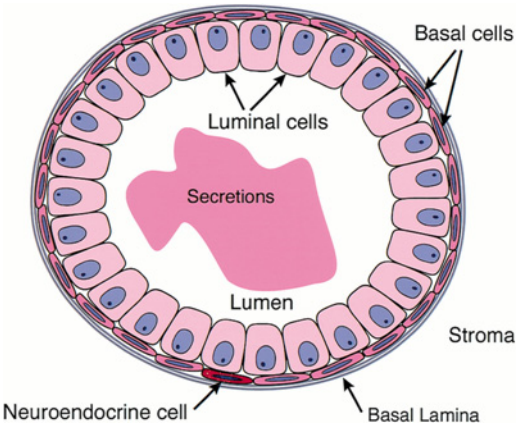

Fig. 1 Schematic view of the cell types within a human prostatic duct. Neuroendocrine cells are morphological indistinguishable from basal cells. Taken from [Abate-Shen, 00].

Prostate cancer progresses from an enlargement (benign prostatic hyperplasia [BPH]) to precursor lesions (prostate intraepithelial neoplasias [PIN]) on to invasive carcinomas and ultimately to metastases. BPH is an abnormal growth of prostate cells occurring in nearly all men over the age of 70. Cells from a BPH are larger in shape but they do not spread to other tissues. Thus benign tumors are not treated unless symptoms such as pain and/or difficulties in the urine flow require treatment. The PIN is a precursor of carcinoma. It is recognized as a continuum between low-grade and high-grade forms with high-grade PIN thought to represent the immediate precursor of early invasive carcinoma [Abate-Shen, 00].

Nowadays there are two major systems used for the pathological graduation of malignant prostate cancers: The Tumor Node Metastasis (TNM), which evaluates the location and size of a tumor and the Gleason grading system, which describes the tumor's degree of differentiation and cell anaplasia. For better understanding of the clinical terminology used in this study a brief description of the main characteristics and pathological classifications of prostate cancer is given in the next paragraphs.

1.4.1 The TNM staging system for prostate cancer

The TNM staging system was already developed in 1977 by Ammon *et al*., but it was not before 1997 that it was predominantly used for evaluating prostate cancer. The TNM staging is based on the location and size of the tumor. It evaluates the local tumor growth (T), the lymph nodes (N) and the distant metastases (M) (Fig. 2). Tumors staged as T1 (a-c) are small and unapparent, they cannot be felt during rectal examination. It may be found by chance when surgery is done for other reasons, usually for BPH (Benign Prostate Hyperplasia). There is no sign that the tumor has spread outside the prostate. A stage 2 tumor is locally restricted tumor, yet lager in size and which can be detected during rectal examination or through biopsy. Tumors stage 3, have spread outside the prostate to nearby tissues such as the seminal vesicles. The last T stage of the TNM system is characterized by tumors which have spread or

are attached to organs near the prostate, such as the bladder. The affection of the lymph nodes is described by pathologist as N status (N0-N3). The M status characterizes the existence of metastasis in organs such as lungs, liver or brain.

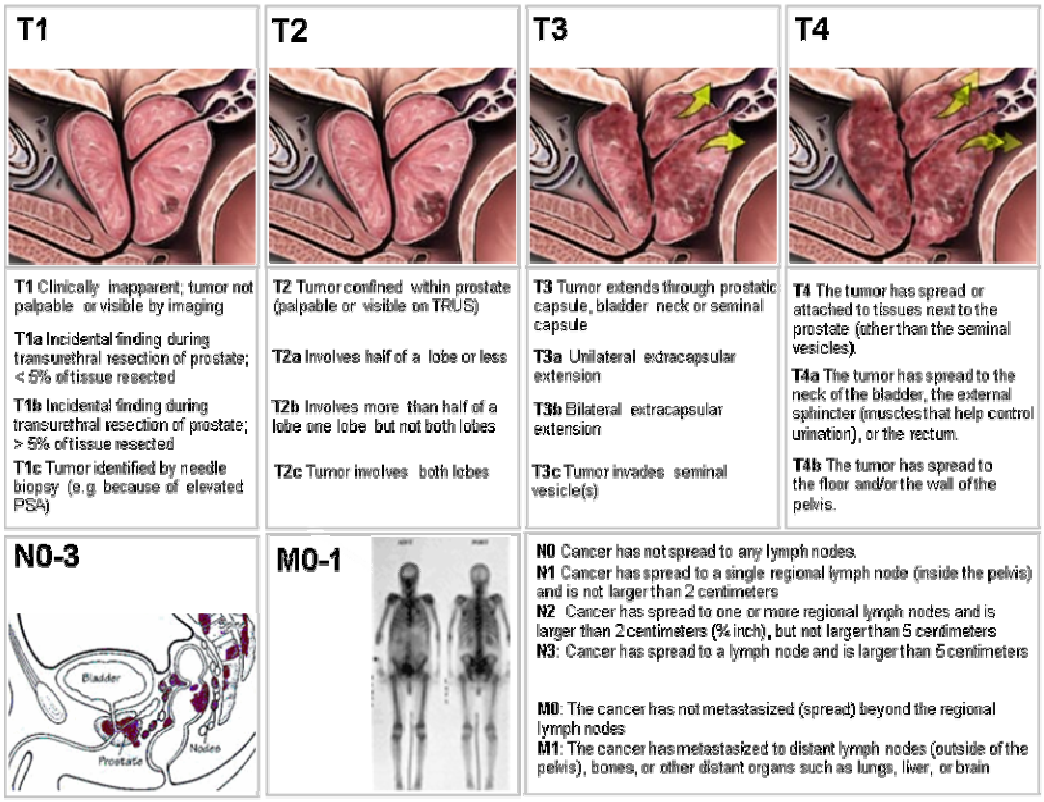

Fig. 2 Anatomical staging of prostate cancer. The TNM system evaluates the location and size of a tumor in the prostate. T = local tumor growth, N = the lymph nodes, M = distant metastases.

1.4.2 The Gleason grading system

In contrast to the TNM system, which evaluates the localization and size of the tumor, the Gleason grading system published by Gleason and Mellinger in 1974 evaluates the tumor's degree of differentiation and cell anaplasia [Gleason, 74] (Fig. 3). Thereby the variation in cell size, shape and staining properties are taken into account. It distinguishes between well differentiated cells, moderately and poorly differentiated cells (cells which are distorted and irregular). The Gleason grading is obtained by summing the degree of cellular differentiation found on the two predominant patterns in a pathological specimen. Well differentiated cells which look closest to normal cells receive the Gleason score 1, poorly differentiated cells get score 5. These two grades are referred to as the Gleason grade. Score 2- 4 is considered as low grade, score 5-7 is considered as moderate, and score 8-10 is considered as a high grade tumor which is poorly differentiated.

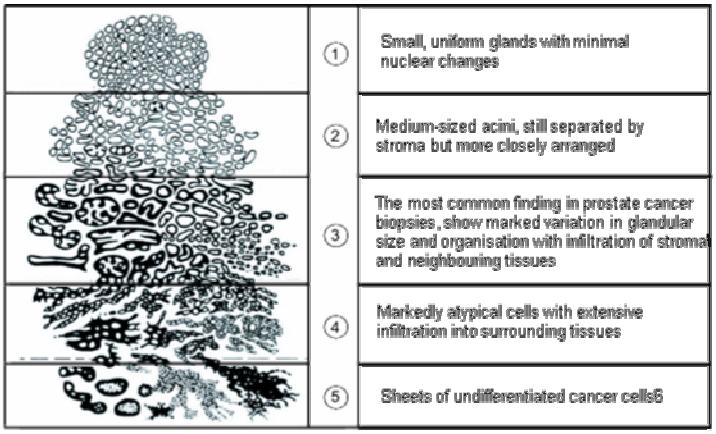

Fig. 3 Gleason Grading of the prostate [Gleason, 74].

1.5 TREATMENT OF PROSTATE CANCER

Although especially in older patients with early stage cancers it is enough to carefully watch the tumor growth as these cancers usually grow at a very slow rate and the possible risks and side effects of therapy may outweigh the possible benefits, many prostate tumors need treated through surgery, radiation or hormones.

1.5.1 Classical treatment

The radical prostatectomy and the radiation therapy are the most commonly used treatment forms for clinically localized prostate cancer (T1 and T2). The surgery involves removal of the entire prostate and in some cases of the surrounding tissues as part of the urethra and the seminal vesicles. Radiation may be used to destroy cancer cells that may have remained in the area after surgery, but it is also used as a stand alone therapy[1].

Tumors which have spread out of the prostate gland (T3) and are thus beyond the reach of a local treatment by surgery or radiation, are treated by hormonal therapy. Although hormonal therapy cannot cure, it usually shrinks or stops the advance of the disease. Drugs which are used nowadays for treatment are either antiandrogens, which block the action of the androgens (for example flutamides and bicalutamide) or drugs which block the testicals from producing testosterone (*e.g.* luteinizing hormone-releasing hormone (LH-RH) agonists as leuprolide and goserelin). Finally aminoglutethimide and ketoconazole are used to prevent the adrenal glands from producing androgens.

Chemotherapy is seldom used for prostate cancer treatment as the response rate is very low. Usually these unspecific systemic drugs are given when hormone therapy has failed. Today drugs such as Docetaxel, Doxorubicin or Estramustine phosphate are used for treatment.

[1] http://www.cancer.gov/cancerinfo/wyntk/prostate

1.5.2 New treatment forms

In the last years the development of target drugs for the treatment of cancers has dramatically increased, a progress that is likely to continue in the future. This approach is based on the targeting of genes found to be overexpressed in tumors or other disease by monoclonal antibodies, small-molecules, immunotoxins and antisense oligonucleotides. This form of therapy has considerable advantage over unspecific systemic drugs such as the chemotherapy. They are more specific, thus less toxic, and more effective in the treatment of cancer [Stockwin, 03]. Antibodies (150 kD) are used to target the extracellular portion of membrane proteins, whereas small-molecules can also inhibit the function of intracellular localized proteins as they can penetrate through the membrane (smaller than 1 kD) [Seemann, 90].

For example antibodies are used to treat indications as diverse as cancer, inflammation and infectious disease. They can be used as cell targeting reagents and thus tag specific cells for complement- or effector-mediated lysis. Antibodies can further be modified to deliver toxic or modulatory payloads (radionuclides or enzymes) [Stockwin, 03].

Up to now several monoclonal antibodies have been developed, especially those gainst the extracellular portion of receptor tyrosine kinases [Seemann, 90]. Herceptin, a humanized monoclonal antibody against the Her2/neu receptor tyrosine kinase, was shown to prolong the survival of women with Her-2/neu positive metastatic breast cancer, when combined with chemotherapy [Seemann, 90].

As an example for a small-molecule the STI-571 has to be named, it was shown to inhibit the Bcr-Abl, c-kit and platelet derived growth factor receptor tyrosine kinases, and thus produced dramatic clinical responses in patients with Bcr-Abl positive chronic myeloid leukemia and c-kit positive gastrointestinal stromal tumors [von Bubnoff, 03].

It seems possible that targeted drugs will be used in association with existing medical, surgical, and radiotherapeutic therapies and will play an important role in the aim of curing cancer.

1.6 ANDROGENS IN PROSTATE CANCER

The importance of androgens in prostate cancer was first described by Huggins and Hodges in the early 1940s [Huggins, 02]. Since then, significant research has shown that the interrelationship between hormone and cancer is very complex and is best exemplified by the recurrence and progression of prostate cancer after hormonal therapy to a lethally resistant phenotype [So, 03].

Androgens, principally testosterone, play a critical role in the development and growth of the male reproductive system. Their biological actions are mediated by the androgen receptor (AR), a ligand-dependent transcription factor, belonging to the nuclear receptor superfamily. These androgen-AR complexes interact with various transcription activators or repressors in order to modulate transcription of

androgen target genes via specific DNA sequences [Lee, 03]. The AR is composed of an N-terminal domain, a DNA binding domain, a hinge region and a ligand binding domain. In its inactive form the AR is complexed to heat-shock proteins in the cytoplasm. After binding a specific ligand (i.e. 5alpha-dihydrotestosterone) the ligand-receptor-complex translocates to the nucleus and binds a specific androgen responsive element (ARE) within the promoter of various genes. Genes affected by the AR are for example the KLK3 (Kallikrein3) gene which codes for PSA and the homeobox gene NKX3-1 [Gregory, 98].

Androgen ablation and anti-androgen therapy has become the cornerstone of treatment for patients with locally advanced or metastatic prostate cancer. Among the earliest detected effects of androgen withdrawal are decreases in the intranuclear concentration of androgens and the AR as well as decreased PSA levels in the blood. Although 80 – 90% of patients respond initially to this therapy the majority gradually develops resistance [Laufer, 00]. The mechanism of change from tumors being androgen-responsive to being androgen-unresponsive is poorly understood [Suzuki, 03]. Interestingly, clinical findings indicated that in androgen ablation therapy-resistant prostate cancer PSA and other genes regulated by androgens as well as the AR by itself are still expressed. This led to the assumption that a ligand-independent activation of the androgen receptor may be the underlying mechanism of androgen independence. In fact, multiple signaling pathways have been implicated in AR non-steroidal activation including estrogen, progesterone, peptide growth factors and cytokines [Debes, 02]. These factors are able to induce transactivation of the AR under androgen-depleted conditions reviewed in[Huang, 02]. Dysregulation of the AR in prostate cancer further results in an abnormal profile of AR-regulated genes which include cell cycle regulators, transcription factors and proteins important for cell survival, lipogenesis, and secretion. Additionally, this receptor is a target for somatic mutation and deregulated androgen signaling is a potential consequence of such mutations reviewed in [Bentel, 96]. Not only is this androgen independence a sign of an emerging disease, it is also associated with a poor prognosis [Sadar, 99].

1.7 DIAGNOSIS OF PROSTATE CANCER

Digital rectal examination (DRE), measurement of the prostate specific antigen (PSA) in the blood and the transrectal ultrasonography (TRUS) are the main parameter used in prostate cancer diagnosis. Nowadays prostate cancer is not diagnosed by symptoms, but because of increased levels of PSA in the blood and abnormal findings in the DRE. Thus it was possible to diagnose more and more patients at earlier stages of the disease, hoping to increase the probability of a cure.

1.7.1 Prostate Specific Antigen

Prostate cancer antigen (PSA) is a tissue specific tumor marker routinely used to diagnose prostate cancer and to monitor treatment response, prognosis and progression of prostate cancer [Sadar, 99]. It is a single-chain glycoprotein with a molecular mass of about 33 kD which functions in the liquefaction of seminal coagulum. Serum levels of PSA of healthy patients are between 0 – 4 ng/ml. In prostate tumor patients the PSA levels can raise up to 100 ng/ml. Generally, PSA levels rise with tumor volume, but it is expressed in all stages of cancer [Caplan, 02]. Although PSA is the best marker for prostate cancer existing today, it is still far from being perfect. For example, PSA tends to increase with age and rises in men with evidence of benign prostatic hyperthrophy. Thus many men are diagnosed falsely positive for prostate cancer. On the other hand PSA levels do not increase in some patients with prostate cancer which leads to a false negative diagnosis. Additionally, preoperative PSA cannot be used to predict capsular penetration or seminal vesicle invasion. Further, PSA is not able to predict progression in adenocarcinomas of the prostate following radical prostatectomy [Sauvageot, 98].

1.7.2 Regulation of PSA

The Kallikrein 3 (KLK3) gene which codes for PSA is primarily regulated by androgens. In the proximal promoter of the KLK3 gene are two functional androgen-response elements (AREs) located [Riegman, 91; Cleutjens, 96]. The core region of the enhancer could be mapped within a 440-bp fragment. A functionally active, high-affinity androgen receptor binding site (GGAACATATTGTATC) was identified in the center of this fragment. Mutation of this element almost completely abolished PSA promoter activity. Therefore PSA levels undergo a sharp decline following an anti-androgen therapy or surgical castration. However, when in the absence of androgens the tumors change to an androgen-independent state, PSA levels increase due to an alternative activation mode. At this stage tumor progression is mostly inexorably and untreatable.

1.8 MICROARRAY ANALYSIS IN CANCER RESEARCH

The main goal of metaGen Pharmaceuticals GmbH was the identification of novel target genes for the development of therapeutic antibodies or small-molecule drugs in different tumor entities. One of the main questions was to find the best method for the identification of new genes. The first description of a “high-capacity system to monitor the expression of many genes in parallel” was published in 1995 [Schena, 95]. They showed that it was possible to detect the expression of 45 Arabidopsis genes simultaneously by spotting the complementary DNAs of these genes on a glass slide and hybridizing samples of RNA to this chip. The development in this field has been more than dramatic in the last years. Today it is possible to detect the expression of the complete

human genome, represented by approximately 47,000 transcripts on only one DNA chip (Affymetrix, Santa Clara, CA, USA).

Nowadays two major groups of DNA microarrays are available: First, cDNA microarrays where oligonucleotides or cDNAs are spotted to a glass microscope slide, second, high density microarrays where nucleotides are synthesized to a specific matrix (Affymetrix). The advantage of the first method is the high flexibility of genes spotted to the slide and the possibility of hybridizing two different samples simultaneously to the chip at relatively low cost. Affymetrix chips only provide the possibility of hybridizing one pool of mRNA at a time at relatively high cost, but have the considerable advantage of synthesizing more than 40,000 genes to one chip. Probe preparation and analysis procedures are quite the same for both chips: The isolated RNA from tissues or cell lines is labeled with fluorochromes before hybridization. A scanner records the intensity of fluorescence per probeset and different bioinformatics tools are used to interpret the huge amount of data sets.

In order to detect genes relevant in different cancer entities metaGen decided to use the Affymetrix technology. At that time it was not possible to synthesize more than approximately 10,000 genes on one chip. Thus 5 chips would have been necessary to analyze the whole genome for differentially expressed genes. First of all this approach was much too expensive and on the other hand, most of the genes present on these chips are not relevant in prostate cancers.

Consequently, a customized Cancer-Chip was designed at metaGen for the identification of tumor specific genes (Fig. 4). The chip design based on a bioinformatic attempt mining systematically expressed sequence tag (EST) libraries [Schmitt, 99]. Briefly, about 4 million ESTs of public[2] and proprietary databases were sorted for tissues specificity and into pairs of benign and cancer tissues. The numbers of ESTs matching to a specific sequence were counted for each pool (normal, tumor and tissue). The sequences which exhibited significant differential expression between normal and cancer tissue were selected and added to the metg001A chip (The procedure is described in detail in “Methods”).

[2] http://www.ncbi.nlm.nih.gov/dbEST/

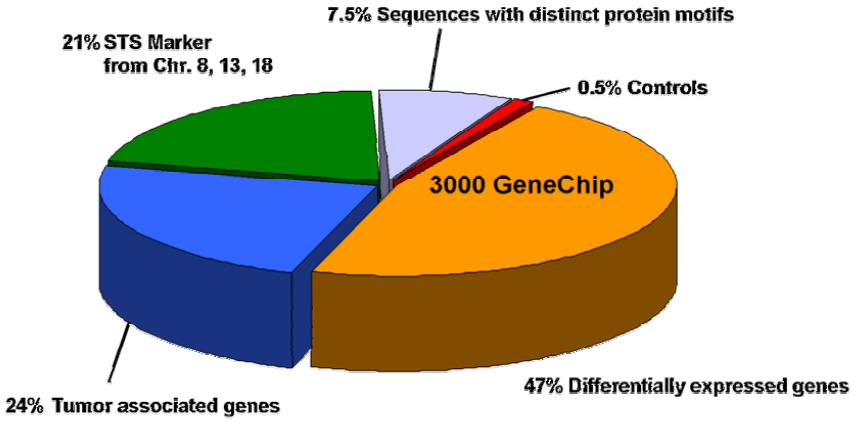

Fig. 4 The metaGen Affymetrix Cancer-Chip (metg001A). This chip contains about 6200 probe sets which represent roughly 3,000 genes. Nearly half of the sequences represent genes which have been shown to be overexpressed in various tumor entities.

These sequences and most of the known tumor associated genes made up the main part of the newly developed metg001A Cancer-Chip. This concentration of cancer associated genes on one chip made it possible to screen for relevant tumor markers in different entities at relatively low cost and in dramatically reduced time. By the use of this proprietary chip it was strongly expected to find overexpressed genes in cancers which have not yet been discovered by other groups and by other methods.

1.9 TRANSIENT RECEPTOR POTENTIAL CHANNELS

Hybridization of the metg001A chip with 52 matched prostate normal and tumor tissues revealed a number of genes differentially expressed in prostate cancer patients. One of these genes, the Transient Receptor Potential Protein 8 (TRPM8), was selected for further evaluation. At the time of identification TRPM8 was a completely unknown gene, not described in the literature.

Up to now, some very interesting features of this gene and especially of this whole protein family have come up. A short overview will be given in the next chapters.

1.9.1 The TRP superfamily

The outstanding feature of the TRP superfamily is its considerable diversity in modes of activation and function. They are involved in processes ranging from sensory physiology of cold and heat to vasorelaxation and male fertility [Montell, 02]. The discovery that TRP channels are able to sense temperatures and flavors was honoured as one of the top ten scientific achievements in the year 2002 by *Sience,* showing the increasing importance of TRPs *[02]*.

Biochemically the TRPs belong to the group of non-voltage gated ion channels - the so called capacitive calcium entry (CCE) channels [Nilius, 03a]. They are activated by various chemical and physical stimuli and also by depletion of

intracellular Ca^{2+} stores, which is followed by a cation influx to the cytosol. TRPs consist of six transmembrane spanning helices, a pore region between Transmembrane (TM) 5 and TM 6 and cytoplasmatic N- and C-termini [Clapham, 01] (Fig. 5).

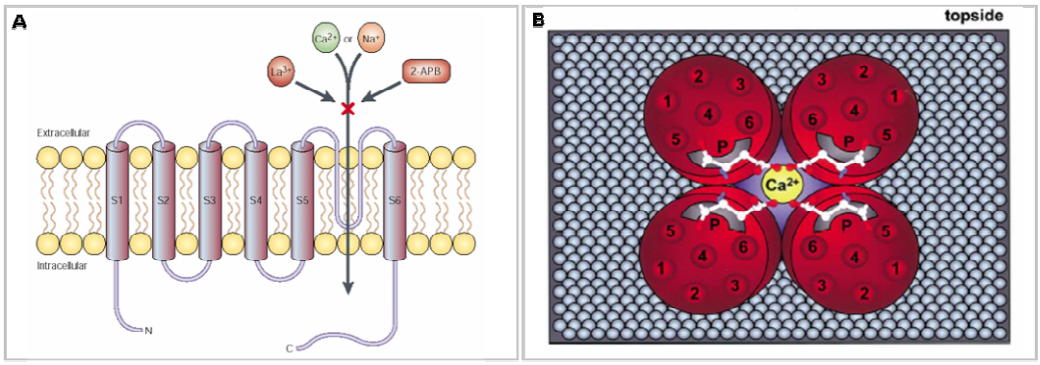

Fig. 5 Architecture of TRP channels. A) TRP channels consist of six transmembrane spanning helices and a pore region between TM5 and TM6 where different mono- and divalent cations can pass through the pore [Clapham, 01]. B) Top view of the TRPV5/6 heterotetrameric channel. The complex is formed by four momomeric subunits of TRPV5/6. The calcium binding site within the pore is formed by 4 aspartate residues [den Dekker, 03].

The first member of the TRP family was identified as a Drosophila gene responsible for visual transduction [Lo, 81]. Because of its transient rather than sustained response to light in mutant flies it was named transient receptor potential (trp). Up to now more than 20 mammalian TRP members are known. They are classified into three subfamilies according to their structural and sequence similarities [Nilius, 03a; Grimm, 03]. The first group are the TRPC (C stands for canonical subfamily) which have a high homology to *Drosophila* TRP channels. Second, the TRPV subfamily (V stands for vanilloid) which are closely related to vanilloid receptor 1 (TRPV1), and third the TRPM family members which are highly homologue to the tumor suppressor melastatin (TRPM1).

The phylogenetic tree of all mammalian TRPs as known today is shown in Fig. 6.

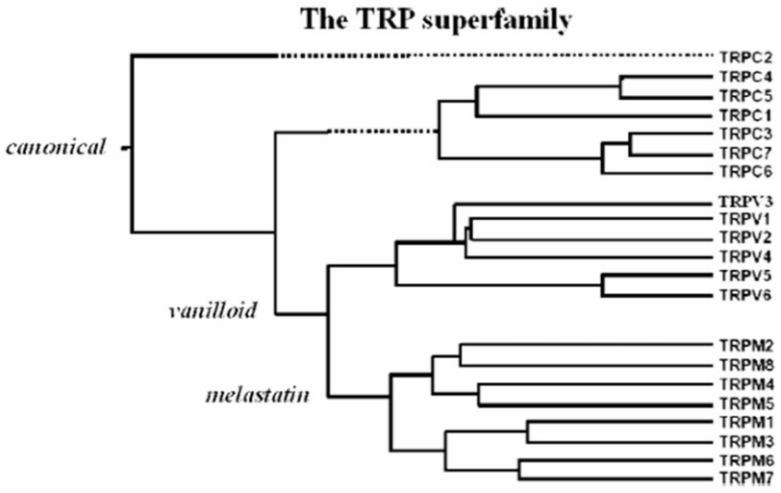

Fig. 6 The phylogentic tree of the mammaliean TRP channels based on their homology [Nilius, 03a].

TRPs are activated mainly through the phospholipase C (PLC) and G-protein coupled receptors (GPCR) which in turn generate inositol (1,4,5) trisphosphate and diacylglycerol (DAG) [Bakowski, 02; Putney, Jr., 97; Putney, Jr., 99]. But also DAG by itself has proved to activate TRP channels [Hofmann, 99; Chyb, 99]. Surprisingly during the last years new TRPs have been indentified which can be activated by chemical and physical stimuli such as heat, cold, mechanical stress, bitter-sweet compounds, reactive oxygen species, pH, pheromones, phorbolesters and vanilloid compounds. For example TRPV1 responds to capsaicin and temperatures over 43°C by generating inward membrane currents, suggesting that it functions as a transducer of painful thermal stimuli [Caterina, 97].These findings are remarkable as these functions give insight into new modes of channel regulation [Nilius, 03a].

1.9.2 TRPM 1 to 7

Less research has been done on members of the TRPM family. Until now 8 homologs of TRPMs are known, counting form TRPM1 to TRPM8. The name (TRPM) has been chosen because the first described member of the group was melastatin (MLSN) [Montell, 02]. Prior to the implementation of a unified nomenclature by Montell at al. in 2002 this subfamily was also known as LTRPC. Members are characterized by relatively long N- and C-termini with some of them having entire enzyme domains linked to their C-terminus. For example TRPM2 has an ADP-ribose pyrophosphatase [Perraud, 01] and TRPM6 and TRPM7 have an atypical α-kinase domain in TRP [Perraud, 01; Runnels, 01; Schlingmann, 02]. Although most of the functions of TRPMs are not known, some TRPM appear to play an important role in cancer and cell proliferation. For example TRPM1 was described as a putative tumor suppressor gene expressed in melanocytes correlating inversely with tumor aggressiveness and the potential for melanoma metastasis [Montell, 02; Duncan, 98]. Another channel, TRPM2, was shown to mediate apoptotic cell death when activated by H_2O_2 in HEK293 cells. This process was accompanied by an increase in intracellular calcium levels ($[Ca^{2+}]_i$) [Duncan, 98; Zhang, 03]. TRPM3 is expressed in human brain and kidney. It is supposed to play a role in the renal homeostasis as it increases Ca^{2+} entry during reduction of extracellular osmolarity [Grimm, 03].

TRPM4 is a remarkable example of the great functional diversity of the TRP protein family. It was shown to be directly activated by cytoplasmatic calcium but the following large inward current is carried primarily by monovalent cations such as Na^+ [Launay, 02]. This shows that TRPM4 is most likely impermeable for calcium. Another striking feature of this protein is that it is the only voltage dependant channel of this group [Nilius, 03b]. TRPM6 was shown to be involved in familial hypomagnesemia being responsible for renal excretion of calcium and magnesium [Walder, 02]. TRPM7 was described as a channel and a protein kinase as well.

1.9.3 TRPM8

TRPM8, formerly known as Trp-p8, is the latest identified gene of all TRP channels. First mentioned in 2001 as a gene upregulated in prostate cancer and other malignancies [Tsavaler, 01] it was shown to be expressed also in a large spectrum of nonprostatic primary cancers such as melanoma, colorectal carcinomas and breast carcinomas. In normal human tissues expression was found mainly in the prostate with trace expressions in testis, breast, thymus and lung.

TRPM8 is closest related to TRPM2 followed by TRPM1 with which it shares 34% sequence identity. Tsavaler et al. suggested that TRPM8 could be an oncogen or tumor promoter gene. It was assumed to belong to the 7-transmembrane proteins.

To describe the importance of TRPM8 for the development of a small-molecule or antibody based therapy is a deal between Dendreon and Genentech from 2002. The agreement "provides for upfront and milestone payments totaling over US $ 110 million for the resulting development of TRPM8 products"[3] .The deal concerns the development of monoclonal antibodies, small molecules and other products derived from Dendreon's TRPM8 gene platform.

In 2002 two manuscripts were published showing the identification of the mouse and rat ortholog of TRPM8 [McKemy, 02; Peier, 02]. Interestingly, both genes were identified in cells from neuronal origin. The mouse ortholog was isolated from newborn dorsal root ganglia and the rats from trigeminal neurons of newborn rats. The striking new discovery was that these channels could be activated by cold and different cooling agents such as menthol, icilin and eucalyptol when overexpressed in cells. Following activation an increase in intracellular calcium was observed which could be suppressed by removal of extracelluar calcium. The authors suggest that TRP channels are the primary transducers of thermal stimuli. Until now 4 heat activated TRP channels and two cold sensing channels have been described: TRPV4 (27-34°C) [Guler, 02], TRPV3 (20-40°C) [Xu, 02; Smith, 02], TRPV1 (>43°C) [Caterina, 97] and TRPV2 (>53°C) [Caterina, 99] TRPM8 (8-28°C) [McKemy, 02] and ANKTM1(12-24°C)[Story, 03].

1.10 CALCIUM SIGNALING

Calcium signals control a wide range of cellular events, ranging from secretion and contraction to gene expression. Calcium can control cell growth and cell differentiation but also induce apoptosis. The concentration of free calcium ions in the cytosol is generally less than 10 µM, a thousand times less than that from the extra cellular space. In general, there are two possibilities for increasing intracellular free calcium ($[Ca^{2+}]_i$) levels: 1. opening of channels located in the plasma membrane allowing calcium ions to move along the electrophysiological

[3] http://www.pharmaventures.com/ag_demo/pr_11144.html

gradient to the interior of the cell. 2. Internal stores as the endoplasmatic reticulum and the sarcoplasmatic reticulum release Ca^{2+} into the cytoplasm. These stores have a limited capacity and must be refilled from the external environment. This process of replenishing is accomplished by store operated Ca^{2+} channels (SOC) which are located in the plasma membrane. They trigger calcium from the external environment through processes known as capacitive Ca^{2+} entry to cell intracellular stores [Hardie, 92; Montell, 97]. This group of calcium channels is named non-voltage-gated channels, because their activation is independent of changes in voltage. Consequently the second large group of Ca^{2+} permeable channels is called voltage-gated channels.

1.11 GENETIC ALTERATIONS OF PROSTATE CANCER

The hypothesis that multiple mutations have to occur before progression from normal to invasive carcinoma can occur has been proven by the identification of multiple chromosomal abnormalities in cancers, including prostate cancer reviewed in [Bova, 96]. In prostate cancer one of the most frequent ly observed chromosomal aberrations is the loss of chromosomal region 8p12-22 [Bova, 93; Macoska, 95; Vocke, 96]. A loss of heterozygosity in this region occurs in 63% of prostate intraepithelial neoplasias (PIN). Although no bona fide tumor suppressor has been mapped to this region, the most probable candidate at this loci is the NKX3-1 gene, which maps to 8p21 [Abate-Shen, 00; He, 97]. NKX3-1 belongs to the large group of homeobox genes. The homeobox sequence encodes a 60-amino acid domain called the homeodomain responsible for DNA binding. These genes are mainly specific nuclear proteins which act as transcription factors. They are the master developmental control genes which regulate cell differentiation and other morphogenic processes [Nunes, 03]. Recent research has demonstrated that deregulation of developemental genes cause cancer. In many cancers such as leukemia, colon, skin, breast, ovary and prostate alterations of gene expression of these genes have been described. For example the PRX homeobox family is strongly connected with human diseases, especially cancer [Silberstein, 02].

1.12 CONTROL OF GENE EXPRESSION

The ability of cells to determine which gene needs to be expressed at a given time and to coordinate transcription is a complex process. Various elements like transcription factors, matrix attachment regions, locus control regions, promoters, gene methylation, enhancers and silencers control the complex transcription machinery [Werner, 03]. First, the chromatin structure of the DNA displays a physical barrier for transcription factors and polymerases to bind to their target DNA sequences [Emerson, 02]. Diverse enzymes modulate the accessibility of DNA by changing the structure of the histones and by modeling the nucleosomes in an ATP-dependent manner. Second and most important for gene expression is the transcription initiation. Transcription requires binding of transcriptional regulatory proteins, RNA polymerases and proteins called

mediators to the promoter region of a gene. A promoter is a region of DNA extending 150-300 base pairs upstream from the transcription start site and represents the central processor of the transcription control. The sites where the transcription factors bind are generally composed of 10 to 30 nucleotides, but usually only a small number of core nucleotides are necessary for binding [Werner, 03].

The complexity and precision of intron removal during mRNA splicing is still an amazing process, although it is known since 1977 [Berget, 77; Chow, 77]. In recent years it has become clear that most human genes express more than one mRNA by alternative splicing [Faustino, 03]. Human genes contain on average 8 exons with an average length of 145 nucleotides. Introns are usually more than 10 times of this size, some are much larger [Lander, 01]. The classical splicing signals at the intron/exons borders are present in 99% of all human introns (Fig. 7). They are necessary for the recognition of exons by the spliceosome, which catalyses the cut-and-paste reactions that removes introns and joins exons [Faustino, 03]. Surrounding these major splicing signals different auxiliary elements such as exonic splicing enhancers or silencers (ESE and ESS) and intronic enhancers of silencers (ISE and ISS) are commonly found. Together with the binding factors these elements are required for efficient splicing of constitutive and alternative exons.

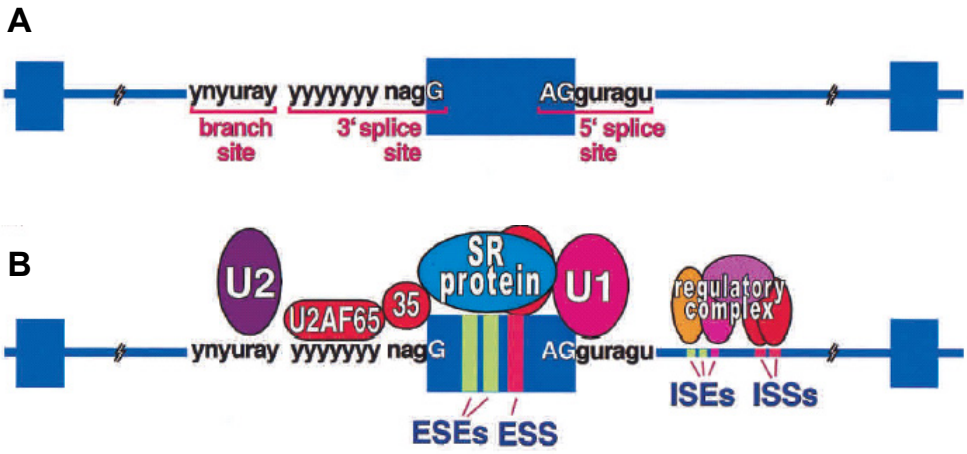

Fig. 7 Classical and auxiliary splicing sites and binding factors taken from [Faustino, 03]. A) Classical and auxiliary splicing site. These sites are found in >99% of the human introns necessary for exon recognition (n = G; A; U, or C; y = pyrimidine; r = purine). B) Classical and auxiliary binding factors. (ISE/ISS = Intronic Splicing Enhancer/Silencer; ESE, ESS = Exonic Splicing Enhancer /Silencer)

Alternative splicing is characterized by the connection of different 5' and 3' splice sites within a gene, resulting in multiple mRNAs expressed by one gene. This process leads to transcripts with one or more skipped exons, variable positions of exons and additions of alternative exons, either within a gene or at its 5' or 3' end. This effect may lead to an expanded protein repertoire which could explain the apparent discrepancy between gene number and the complexity of higher eukaryotes [Mercatante, 02]. Up to 59% of human genes were found to

be spliced alternatively [Lander, 01] and ~ 80% of these splicing results in an altered protein [Modrek, 02]. In most cases regulation of alternative gene expression is cell type specific. The regulation is mediated by intronic repressors or activator elements distinct from the classical splicing sequences. Expression of these alternative mRNA forms was seen during specific stages of development, in specific cells or tissues as well as in numerous diseases including cancer [Mercatante, 02].

Until now four classes of mRNA splicing (two *cis* acting and two *trans* acting) leading to a disease have been described reviewed in [Faustino, 03]: First and most common are mutations in the constitutive splice sites. These can lead to unnatural mRNAs which are deleted by a nonsense-mediated decay or to the loss of function of the resulting protein (protein instability, truncations). Second the disruption of alternative splice sites which have been described for 4 diseases (Familial isolated growth hormone deficiency type II- caused by mutations in the growth hormone gene; Frasier syndrome - caused by mutations in the WT-1 gene; Frontotemporal dementia and Parkinsonism linked to Chromosome 17 - caused by mutations in the MAPT gene and the atypical cystic fibrosis - caused by polymorphisms of the CFTR gene) The two *trans*-acting classes are characterized by mutations either in the basal splicing machinery or mutation in factors regulating the alternative splicing machinery. The major group of splicing factors are the serine/arginine-rich (SR) proteins which are members of a conserved family of proteins that bind to the active sites of RNA polymerase II and thus function as key regulators of alternative RNA splicing [Zahler, 92; Fu, 95; Graveley, 00]. They have dual functions and serve as splicing enhancer or splicing repressor proteins, depending on where in the pre-mRNA they bind reviewed in [Akusjarvi, 03].

Aberrations in alternative splicing were found as a contributing factor or cause to the development, progression or maintenance of cancer. Up to now there are only models of how this process is regulated. Most likely specific repression of activation complexes surrounding the regulated splice sites serve to enhance or inhibit the recognition of the classical splice sites by the basic splicing machinery [Charlet, 02]. But it remains unclear why the expression of different transcripts is enhanced in one tissue, whereas it is repressed in others. One interesting question is, if the altered expression of splice variants in cancer is a cause for the disease (i.e. due to mutations within the auxiliary elements within exons or introns) or the effect from other disordered genes which might be involved in the splicing machinery.

1.13 AIM OF THE THESIS

The aim of this thesis was the identification of new genes differentially expressed in prostate tumors. Therefore microdissected matched prostate cancer and benign tissues of 52 prostate cancer patients were hybridized to a proprietary high density Cancer-Chip based on Affymetrix GeneChip technology. The intention was to identify genes differentially expressed in prostate cancers which had not yet been discovered by other groups. One of these genes was then selected for a more detailed analysis.

In order to answer, whether the selected gene could be used as a target for a small-molecule or antibody based therapy the following questions were aimed to be answered.

1. Is the gene expressed in other cancer entities than the prostate?
2. In which normal tissues is it expressed?
3. Does it correlate with prostate cancer progression?
4. In which cell compartiment(s) is the gene expressed?
5. Is the gene an oncogene?
6. How is it regulated?
7. Is the gene also useful for diagnostics or other possible cancer therapies?

The answers should provide evidence whether it is worth to develope a specific drug against this gene or not.

2 RESULTS

2.1 GENE CHIP EXPRESSION ANALYSIS OF PROSTATE CANCER PATIENTS

High throughput gene expression profiling has become an outstanding method for identifying genes differentially expressed in normal and diseased tissue. In order to identify new genes differentially expressed in cancerous tissue, metaGen Pharmaceuticals GmbH designed a customized DNA Cancer-Chip based on Affymetrix technology (Affymetrix, Inc. USA). This oligonucleotide microarray contained 6200 probe sets representing approximately 3,000 genes. The chip design based on a bioinformatic attempt mining approximately 4 million expressed sequence tags (ESTs) of public[4] and proprietary databases [Schmitt, 99]. These ESTs were sorted for tissues specificity and into pairs of benign and cancer tissues. The numbers of ESTs matching to a specific sequence were counted for each pool (normal, tumor and tissue). The sequences which exhibited significant differential expression between normal and cancer tissue were selected and added to the metg001A chip. This selection and concentration of genes relevant in tumors on the metg001A chip was expected to increase immensely the percentage of genes differentially expressed in tumors. Additionally, it was expected to identify new genes, which were not yet discovered by other groups and methods.

Therefore 52 prostate tumor samples and its corresponding normal tissues were collected at the time of radical prostatectomy (RP) at the Department of Urology at the University Hospital Charité from 1999 to 2000. All samples were microdissected and hybridized to the metg001A microarray. Microdissection was used in order to specifically select tumor areas and normal glands to further increase the number of identified differentially expressed genes, especially as prostate tumors are among the most heterogeneous of cancers [Singh, 02]. The clinical and pathological features as well as the follow-up data of all patients hybridized to the chip are listed in Tab. 7 of the attachment.

Analysis of hybridization experiments was done according to the metaGen criteria, described in “Methods”. Briefly, a gene was called differentially when it was significantly expressed in both normal and tumor tissue (p-value < 0.05) and the quotient (fold change [FC]) of the normalized perfect match quintile (PMQ) value of each patient and probe set (tumor/normal) was > 2. For genes present only in normal or tumor tissue (p-value > 0.05) no fold change was calculated, but marked as differentially expressed (FC was set to 2). Finally, genes were ranked according to their differentially expression between normal and tumor tissue.

[4] http://www.ncbi.nlm.nih.gov/dbEST/

Tab. 1 Upregulated genes in prostate cancer patients identified by Affymetrix GeneChip experiments. Genes were sorted by numbers of patients with a fold change > 2 (quotient of PMQ-values of matched tumor and normal sample for each gene), HGN for HUGO Gene Nomenclature, FC for fold change.

HGNC	Description	Lokalisation	FC < 05	FC > 2	FC > 4	Median FC
PCA3	prostate cancer antigen 3	9q21-q22	1	40	34	10,20
TRGC2	T cell receptor gamma constant 2	7p15	2	32	17	3,30
ABCC4	Homo sapiens ATP-binding cassette sub-family C (CFTR/MRP) member	13q32	1	32	13	2,40
TRPM8	Homo sapiens transient receptor potential cation channel subfamily	2q37.2	1	30	6	2,20
OR51E2	Homo sapiens olfactory receptor family 51 subfamily E member 2	11p15	4	29	18	2,30
GOLPH2	Homo sapiens golgi phosphoprotein 2 (GOLPH2) mRNA.	9q21.33	2	28	10	2,10
AGR2	Homo sapiens anterior gradient 2 homolog (Xenepus laevis) (AGR2)	7p21.3	1	27	7	2,00
FOLH1	Homo sapiens folate hydrolase (prostate-specific membrane antigen)	11p11.2	6	27	18	2,20
FASN	Homo sapiens fatty acid synthase (FASN) mRNA.	7q25	3	24	4	1,80
PRAC	small nuclear protein PRAC	17q21	2	23	3	2,00

Microarray analysis of prostate cancer patients revealed that 26% of the probesets (1434 probesets) present on the microarray showed a differential expression in at least one patient. Two criteria were applied for the identification of possible target genes. First, the gene should be overexpressed in tumors in at least 30% of the patients. Second, this gene should not be downregulated in more than 10% of the patients. Following these criteria 38 genes could be identified as overexpressed in prostate tumor patients. From these genes, seven were even upregulated in more than 50% (27 patients) of the tumors. The list of overexpressed genes in prostate tumor patients is shown in Tab. 1.

Tab. 2 Downregulated genes in prostate cancer patients identified by Affymetrix GeneChip experiments. Genes were sorted by numbers of patients with a fold change < 0.5 (quotient of PMQ-values of matched normal and tumor sample for each gene), HGN for HUGO Gene Nomenclature, FC for fold change.

HGNC	Description	Lokalisation	FC < 05	FC > 2	FC > 4	Median FC
KRT15	keratin 15	17q21.2	37	1	1	0,35
PGM5	Phosphoglucomutase 5	9q21.11	30	2	0	0,39
NT_021877	?	1q32.2	29	1	1	0,35
PENK	proenkephalin	8q23-q24	28	1	0	0,28
SERPINB5	serine (or cysteine) proteinase inhibitor	18q21.3	27	1	1	0,45
FGFR2	fibroblast growth factor receptor 2	10q26	26	0	0	0,49
KRT5	keratin 5	12q12-q13	26	2	1	0,50
PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	7q21.3-q22.1	25	2	0	0,49
CCK	cholecystokinin	3p22-p21.3	24	0	0	0,38
CLU	clusterin (testosterone-repressed prostate message 2)	8p21-p12	24	1	0	0,50

62 genes could be identified being underexpressed in at least 30% of the patients (FC < 0.5) using the same criteria as for genes downregulated in prostate cancer.

From these, 5 genes could be identified to be downregulated in more than 50% of the tumors, listed in Tab. 2.

For a more detailed analysis of this prostate cancer profiling, please refer to the thesis of Christoph Wissmann [Wissmann, 02].

2.1.1 Expression of TRPM8 in prostate cancers

After the identification of genes upregulated in prostate cancer, the next task was to find out which genes would be the best targets for an antibody or small-molecule therapy. The following criteria were set up for genes to be further evaluated: 1. The gene had to be upregulated in at least 30% of all tumors, while downregulation should be less than 10%. 2. The gene had to be new (neither mentioned in the literature nor in patent applications nor filed patents) 3. The gene products should be drugable with preference for proteins located in the plasma membrane. Surprisingly not many genes could be found fulfilling these criteria. One of them was the protein standing on position four of the list of genes upregulated in prostate cancer: TRPM8. It was expressed significantly ($p < 0.05$) in 103 of the 104 specimens and it was overexpressed in 56% of all tumor patients with a fold change > 2. ($p < 0.000001$) (Fig. 8). Only in one patient TRPM8 was downregulated in tumors (FC < 0.5).

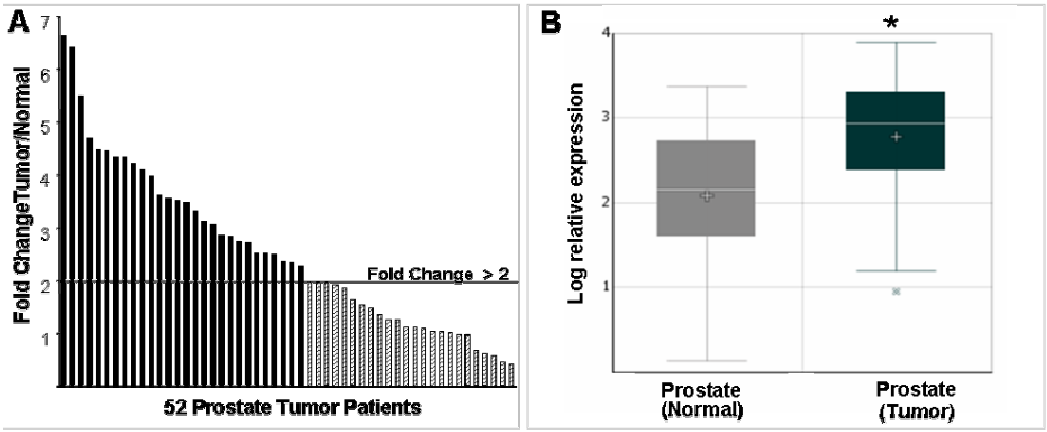

Fig. 8 Affymetrix microarray analysis of TRPM8 expression in matched tumor and normal prostatic tissues. A) Changefold of PMQ values of 52 matched prostate cancer and normal tissues. **B)** Boxplots of PMQ-values of TRPM8 expression of prostate tumor patients grouped into normal and tumor. TRPM8 is significantly overexpressed in prostate tumors ($p < 0.000001$).

At the time of data analysis, TRPM8 was completely unknown and database search of the partial cDNA of TRPM8 revealed a high homology to the transient receptor potential (TRP) family of non-voltage-gated cation channels.

2.1.2 Expression of TRPM8 in human cell lines

In order to characterize the expression of TRPM8 among various cell lines 30 established human cell lines were hybridized to the metg001A chip. These cell lines were derived from normal and cancer tissues from the prostate, bladder, colon, mammary gland, lung and pancreas (Fig. 9). Results indicated that

TRPM8 is exclusively expressed in prostate tumor cell line LNCaP ($p<0.05$). No significant TRPM8 expression could be detected in any other cell lines tested.

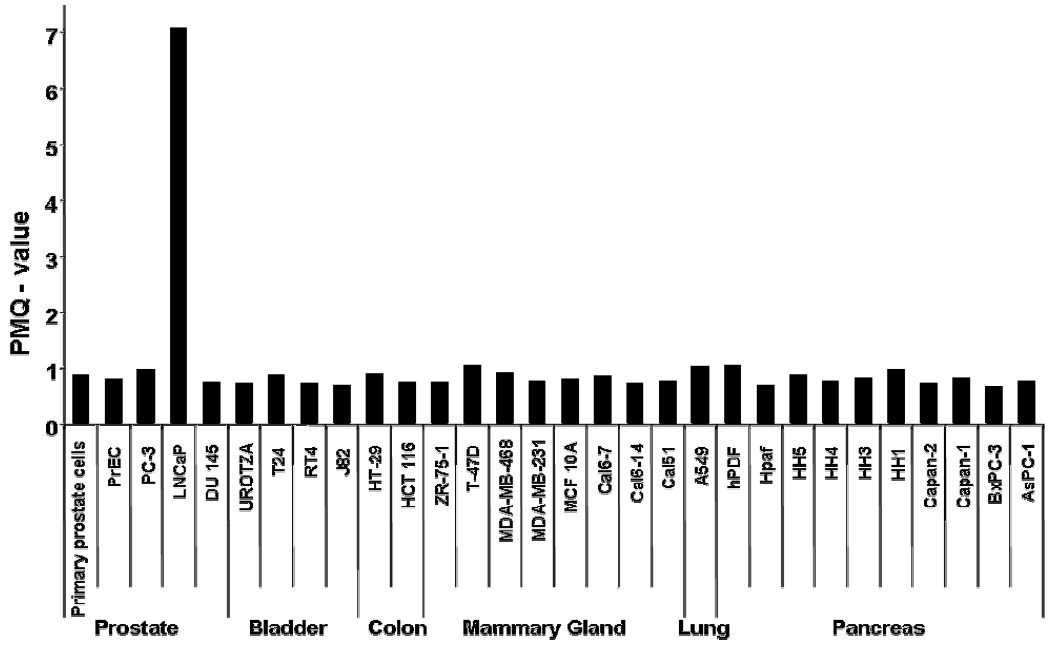

Fig. 9 Affymetrix GeneChip analysis of TRPM8 expression in different cell lines. PMQ values of TRPM8 mRNA expression in 30 cell lines dived from six different tissues including prostate, bladder, colon, mammary gland, lung and pancreas.

2.2 TRPM8 EXPRESSION IN HUMAN TISSUES

Affymetrix gene chip experiments and electronic Northern analysis revealed that TRPM8 expression is restricted to the prostate. To confirm these high-throughput methods and to analyze the distribution of TRPM8 expression in "wet" experiments, classical Northern blots and dot blots as well as Real Time PCR were performed on a large number of patient and tissues.

2.2.1 Electronic Northern of TRPM8

In order to use a gene as a target for a therapeutic approach it has to be shown, that its expression is restricted to the tissue (and disease state) to be targeted, or at least, it should not be expressed in organs essentially for survival such as heart or brain. Ideally, the expression of the therapeutic target gene should be restricted to the tissue of interest. Thus, the drug - its specificity presumed - would not affect any healthy tissue and side effects could be minimized.

	NORMAL FREQ	POOLSIZ	TUMOR FREQ	POOLSIZ	RATIO N/T	P-VAL	SIG
Adrenal_Gland	0	25886	0	20230	-	-	-
B-Lymphoma	0	108530	0	7357	-	-	-
Bladder	0	25641	0	42550	-	-	-
Breast	0	117167	0	71112	-	-	-
Colon	0	31042	0	35626	-	-	-
Endometrium	0	18977	0	6023	-	-	-
Glial_Tissue	0	110439	0	38567	-	-	-
Heart	0	98498	0	7271	-	-	-
Kidney	0	44566	0	40078	-	-	-
Lung	0	96193	0	43114	-	-	-
Muscle-Skeleton	0	58315	0	27063	-	-	-
Myometrium	0	35843	0	14713	-	-	-
Ovary	0	15293	0	32525	-	-	-
PBL	0	130844	0	941	-	-	-
Pancreas	0	65807	0	21808	-	-	-
Prostate	**5**	**88661**	**19**	**76762**	**0,23**	**0,00158**	**99,8**
Skin	0	27182	0	1180	-	-	-
Small_Intestine	0	36432	0	9379	-	-	-
Squamous_Cells	0	33833	0	25706	-	-	-
Stomach-Esophagus	0	13799	0	12119	-	-	-
T-Lymphoma	0	60680	0	17313	-	-	-
Testis	0	29540	0	16891	-	-	-
All Tissues	5	1273166	19	568328	0,12	0,0000015	100

Fig. 10 Electronic Northern analysis of TRPM8 expression in 22 human tissues. FREQ = Frequency of a TRPM8-EST in a pool ESTs derived from either normal or cancer tissues N = Normal tissue, T = Tumor tissue, P-val = p-value, Sig = Significance.

Therefore the TRPM8 expression pattern among diverse normal tissues was examined by electronic Northern (Fig. 10). This *in silico* approach is a very effective tool to examine expression patterns of genes among divers tissues. Using the proprietary data set of metaGen approximately 4 million ESTs could be analyzed for the expression of TRPM8. Analysis showed impressively that TRPM8 expression among 22 human tissues was significantly restricted to the prostate. (Significance = 100%; p-value < 10^{-5}). This is one of the rare events that a gene is solely expressed in one organ. Further it could be shown that TRPM8 is highly differential expressed between tumor and normal prostate tissues with a significance of 99.8 and a p-value of 0.00158.

2.2.2 Northern and Dot blot analysis of TRPM8

The probe specific for TRPM8 comprised the first 2700 base pairs of the TRPM8 open reading frame (ORF). Hybridizing this probe to a Northern blot from Clontech (Heidelberg, Germany) representing 16 different human normal tissues revealed that TRPM8 expression is restricted completely to the prostate (Fig. 11). Interestingly, besides the expected 5.6 kb fragment two other transcripts of approximately 7.3 and 4.1 kb could be detected.

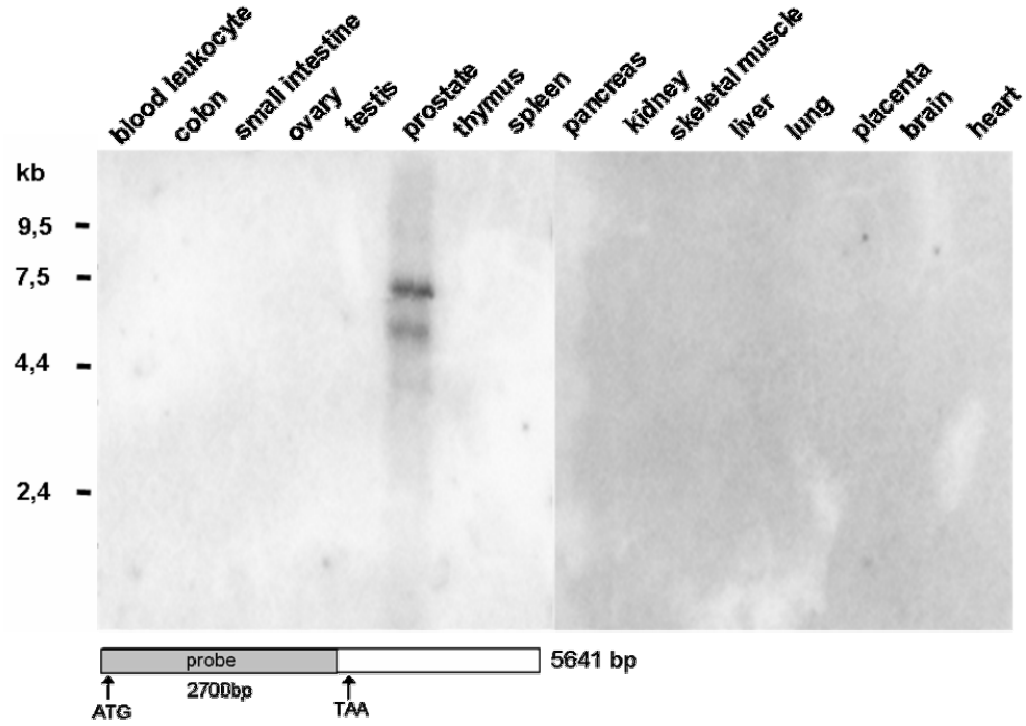

Fig. 11 Northern blot analysis of TRPM8 expression in various normal human tissues (Clontech, Heidelberg, Germany). The 5'-probe was 32P-labeled and hybridized to the membrane. TRPM8 is expressed exclusively in prostate with different transcripts sizes of approximately 7.3 kb, 5.6 kb and 4.1kb.

Hybridizing the same probe to a Cancer Profiling Array (CPA) (Clontech) which represents cDNA dots of 241 matched normal and tumor tissues of 14 human cancer entities and 9 cancer cell lines confirms the results seen in electronic Northern analysis. TRPM8 is exclusively expressed in the prostate with the exemption of one kidney tumor (Fig. 12).

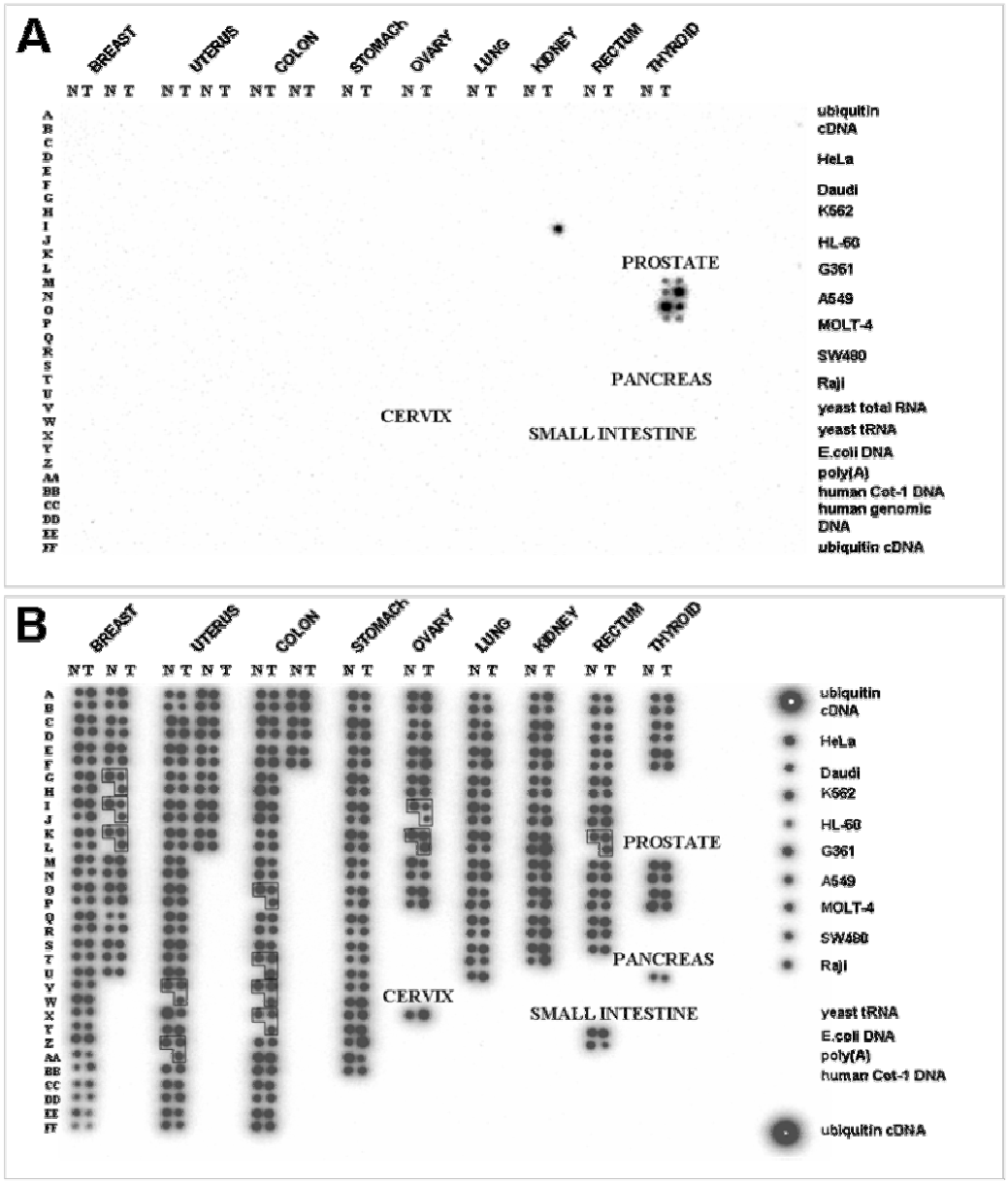

Fig. 12 Cancer Profiling Array representing 241 matched tumor and normal human tissues from 13 cancer entities and several cell lines. A) The TRPM8 specific probe was 32P-labeled and hybridized to the membrane. **B)** The Ubiquitin specific probe was 32P-labeled and hybridized subsequently to the same blot.

2.2.3 Real Time PCR of TRPM8 in matched prostate cancer patients

Quantitative Real Time PCR of TRPM8 expression was done on 42 cDNA from matched tumor and normal prostate cancer patients. Results show that TRPM8 is overexpressed in 64% of prostate tumor patients (Fig. 13**)**. In some patients the relative expression levels of tumor versus normal tissue are very high (20-100 x) which indicates the remarkable overexpression of TRPM8 in prostate tumors. In comparison to microarray experiments Real Time PCR are much more sensitive.

These experiments confirm the results gained in gene chip and dot blot experiments.

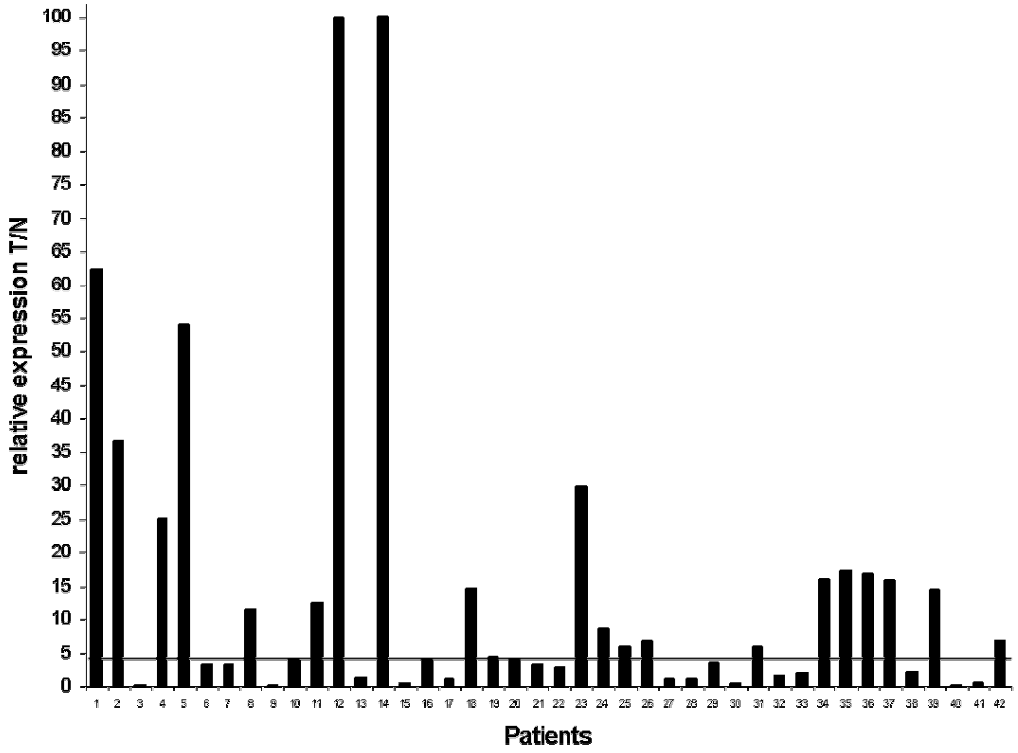

Fig. 13 Relative expression of TRPM8 mRNA in 42 prostate tumor samples by RT-PCR. Data is shown as relative expression levels from matched tumor and normal prostate tissues. T = tumor, N = normal

2.2.4 In situ hybridization of TRPM8 in prostate tumors and other entities

In situ hybridization of TRPM8 expression was performed on 60 normal, 23 prostatic intraepithelial neoplasia (PIN) and 91 adenocarcinomas (Gleason Grading 1-10) of the prostate. Additionally, 10 specimens of each tissue from tumors of the mammary gland, ovary, liver, pancreas and bladder were tested for TRPM8 expression. Expression could be detected in 37 of the 60 normal tissues, in 17 of the 23 PINs and in 75 of the 91 adenoncarcinomas of the prostate. All other tissues remained negative. In normal prostate sections, the epithelial cells showed moderately positive hybridizations signals. Strongest signals however were observed in the epithelial cells forming the lumen of the duct (Fig. 14). Smooth muscle cells and connective tissue remained negative. Relative expression levels comparing normal and tumor samples from the prostate samples showed an upregulation in 28 of 56 matched tumor and normal samples. TRPM8 expression correlated positively with disease progression from normal over PIN to low grade tumors, but TRPM8 expression was lost in completely undifferentiated tumor cells (Gleason Grading 9-10). This could be observed

especially in cases were high grade tumors and low grade tumors were present in the same section of a specimen.

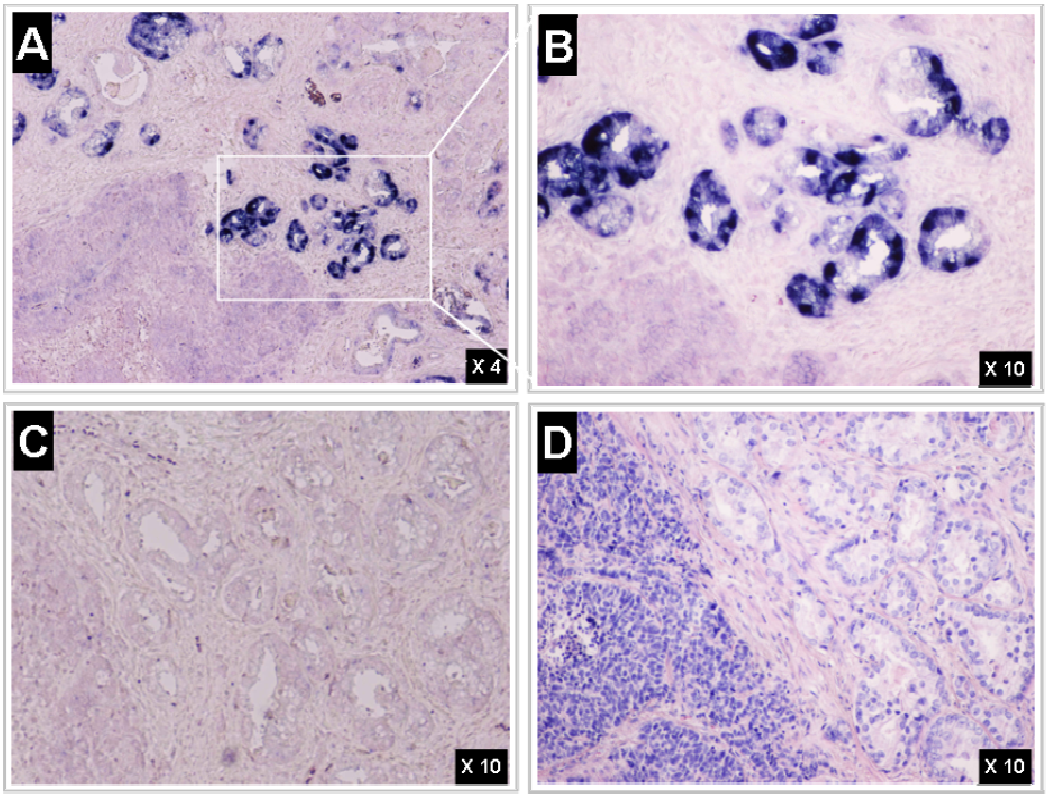

Fig. 14 ***In situ*** **hybridization of TRPM8 mRNA to a prostate adenocarcinoma.** 4 µm sections were hybridized with the TRPM8 probe used in Northern and dot blot experiments. **A)** and **B)** hybridization of the antisense TRPM8 probe to an adenocarcinoma of the prostate. **C)** sense probe of TRPM8 and **D)** Haematoxilin and Elaun staining of the same patient.

2.3 FISH EXPERIMENTS OF TRPM8 ON 2.Q37.2 IN LNCAP CELLS

Fluorescence in situ hybridization (FISH) analysis of the genomic region of TRPM8 was performed to gain insight into the molecular mechanism of the overexpression of TRPM8 in prostate tumors and LNCaP cells. As TRPM8 is located at the very end of chromosome 2 (2q37.2), the chromosomal region could possibly by either amplified and/or translocated to other chromosomes. Through translocation, the TRPM8 gene might get under the control of an alternative promoter or enhancer resulting in an altered expression profile. Both chromosomal amplification and translocation are known mechanisms often occurring in tumor tissues [Nowell, 97]. In order to address this question FISH experiments were performed on LNCaP cells. This cell line was chosen, as it is the only cell line expressing TRPM8. The BAC AC005538 (2q37.2) was used as a TRPM8 specific probe for hybridization. As a positive control a commercially available probe for identifying MYC on 8q24.12-q24.13 was used (Vysis Inc., Downers Grove; IL, USA). A comparison of hybridization signals of TRPM8

between LNCaP cells to normal human XY specimens showed that TRPM8 hybridized exclusively to the expected position on chromosome 2, in both the LNCaP cell and the XY patient (Fig. 15). No hybridization signal could be detected anywhere else in the chromosomes.

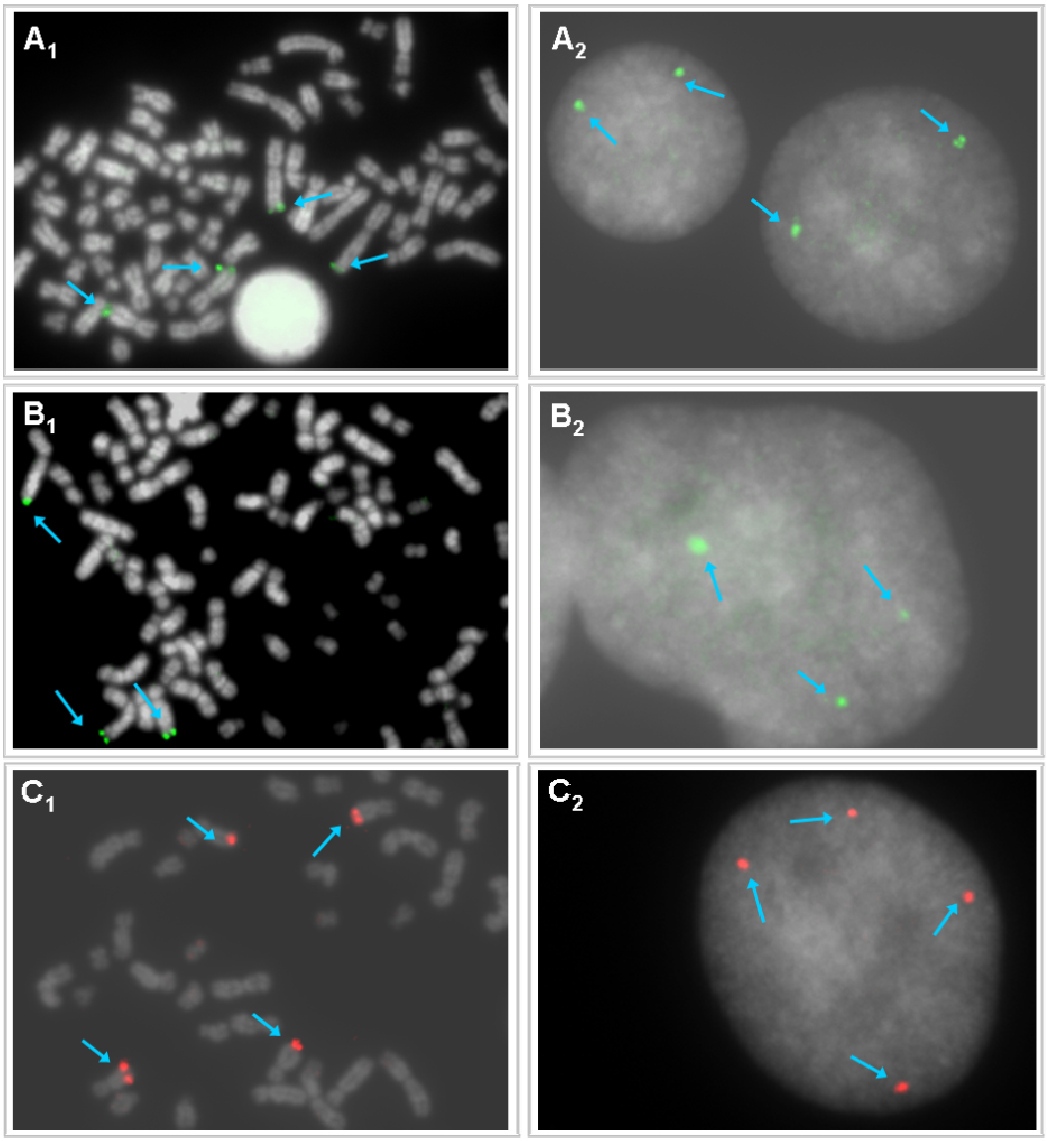

Fig. 15 FISH mapping of the genomic region of TRPM8 and MYC in normal human XY patients and LNCaP cells. Hybridization signals for TRPM8 (AC005538 on 2q37.2.) are shown in green; hybridization signals for MYC (8q24.12-q24.13) are indicated in red. Picture A1 to C1 show metaphase chromosomes. Pictures A2 to C2 show interphases of the same sample. **A)** 1 and 2 show hybridization signals specific for TRPM8 in a healthy XY-person. **B)** 1 and 2 show mapping of TRPM8 in LNCaP cells. **C)** 1 and 2 show the hybridization signal of MYC to chromosome 8.

The finding that chromosome 2 is tripled in LNCaP cells and chromosome 8 (MYC-specific binding) is quadruplicated, is in coherence with the literature [Augustus, 03]. A spectral karyotyping of LNCaP cells shows a predominantly tetraploid karyotype of LNCaP cells. Only chromosome 2 (triploid), 6 (diploid), 19 (triploid) and 21 (triploid) differ from that pattern (Fig. 16).

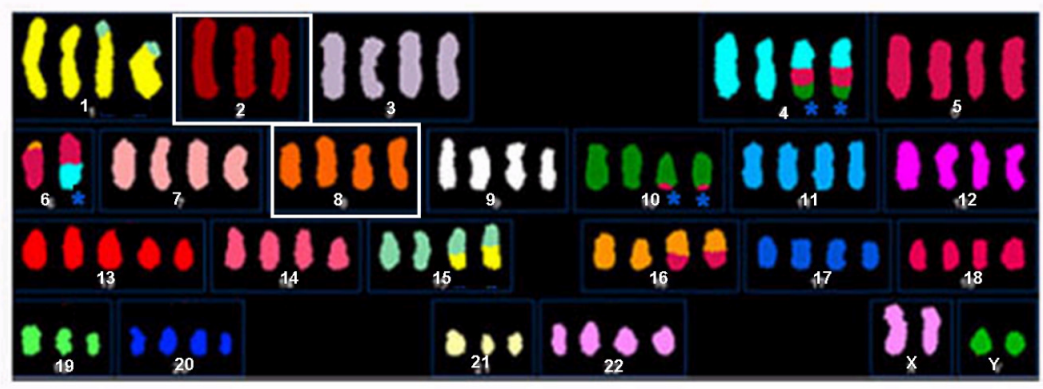

Fig. 16 Spectral karyotyping (SKY) of LNCaP cells taken from [Augustus, 03]. Chromosome 2 and 8 are boxed as these are the chromsomes where TRPM8 and MYC are localized, respectively.

Additionally, the signal strength was not enhanced in LNCaP cells compared to normal XY patients. Therefore, it can be concluded that the overexpression of TRPM8 in LNCaP cells and thus most likely also in prostate tumor patients is not due to chromosomal amplifications or rearrangements.

2.4 HOMOMULTIMERIZATION OF TRPM8 SUBUNTIS

TRPs are known to form either homo- or hetero-tetrames. In order to prove direct interactions between TRPM8 subunits, channel multimerization was assessed using the fluorescence resonance energy transport (FRET) technology. Therefore TRPM8 was terminally fused to CFP and YFP vector construct. These plasmids where transiently coexpressed in HEK293 cells and the proximity of the homomerization was measured with FRET. FRET signal was obtained by measuring the increase in fluorescence of donor (CFP) emission during photobleach of the acceptor (YFP). The recovery of donor fluorescence emission was then monitored at 480 nm and was expressed as percentage of CFP emission after acceptor bleach. The FRET analysis showed a strong interaction of TRPM8 homomultimers which is shown in FRET efficiencies of 15.3% (Fig. 17, A).

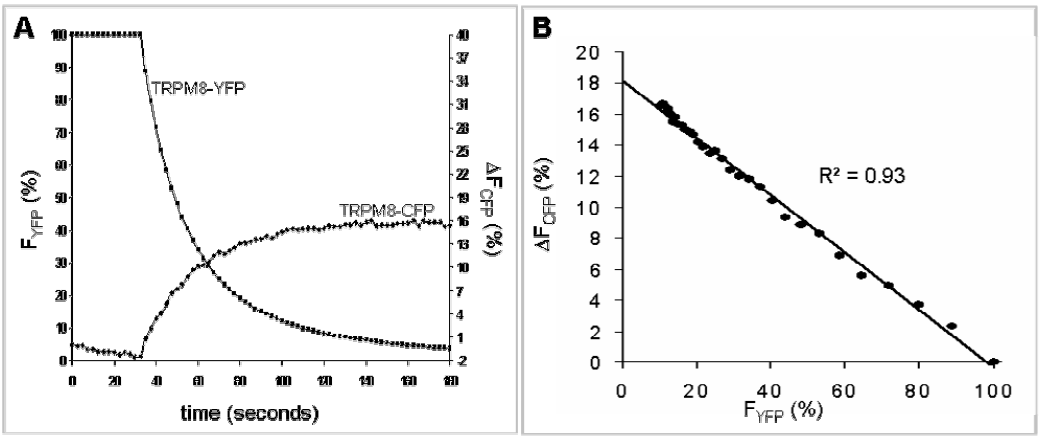

Fig. 17 FRET analysis of transiently transfected HEK293 with TRPM8-CFP and TRPM8-YFP after 24 h. A) and **B)** Recovery of fluorescence intensity of the FRET donor (ΔFCFP) during disruption of energy transfer by photobleaching of the acceptor (FYFP). The acceptor was selectively bleached at ƛ = 515 nm. The relative increase of CFP (ΔFCFP (%) intensities compared to initial levels and YFP fluorescence intensity decrease (FYFP (%)) over time. The increase of CFP fluorescence intensity of 15, 3% is a direct evidence of FRET. Data shown are representative for several FRET analyses.

2.5 ACTIVATION OF TRPM8 BY THE COOLING AGENT ICILIN

It was shown by McKemy *et al*., that the TRPM8 rat orthologue CMR1 can be activated by cold inducing agents such as icilin, menthol and eucalyptol as well as by temperatures below 28 °C resulting in an increase of intracellular calcium levels. The mouse orthologue of TRPM8 could also be activated by menthol and temperatures ranging from 25°C to 15°C [Peier, 02]. In order to find out whether human TRPM8 could also be activated with different cooling agents, TRPM8-stable expressing HEK293 cells were loaded with the Ca^{2+}- indicator Fura-2 and exposed to 50 µM of Icilin. These experiments were done in cooperation with Stefan Mergler from the Charité in Berlin.

As displayed in Fig. 18 (A, B) TRPM8 expressing cells showed a threefold increase in intracellular calcium upon stimulation with Icilin. That response was not observed in nontransfected and empty vector transfected cells. The icilin mediated calcium influx occurred within msec. Washout of icilin induced recurrence of $[Ca^{2+}]_i$ back to levels of unstimulated cells (recovery effect). The response was dependent on Ca^{2+} in the buffer, because removal of extracellular calcium suppressed the Icilin response. The result indicated that TRPM8 is localized in the plasma membrane, although transmembrane localization could not be seen in most of the TRPM8-expressing HEK293 cells. This is most likely due to limitations of sensitivity in the cytochemistry procedures.

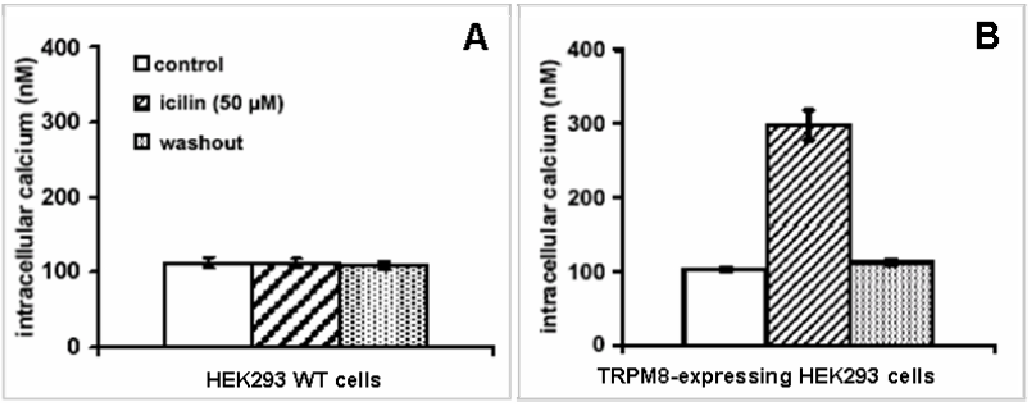

Fig. 18 Analysis of calcium influx of HEK293 cells expressing TRPM8 using Fura-2. Representative measurement data were taken from calcium experiments of transfected and nontransfected HEK293 cells loaded with Fura-2. WT = wild type

2.6 GENE CHIP ANALYSIS OF TRPM8 EXPRESSION IN 7 HUMAN TISSUES

As prostate was the first entity to be hybridized to Affymetrix GeneChips at metaGen, it was interesting to check two years later the expression on TRPM8 in entities other than the prostate. Until today 6 other tissues have been hybridized to several Affymetrix chips (either proprietary or custom designed). Bladder, ovary, mammary gland and pancreas were hybridized to the metg001A Cancer-Chip; lung and colon were hybridized to the U133A and B Affymetrix GeneChip. The probe sets on the U133B were not identical to the ones on the metg001A chip but adequate, as described in detail in "Methods". Bioinformatic analysis of these 7 tissues revealed that TRPM8 expression was restricted to the prostate with two exceptions. One bladder-tumor sample (out of 79) and one lung cancer (out of 172) expressed TRPM8 (Fig. 19).

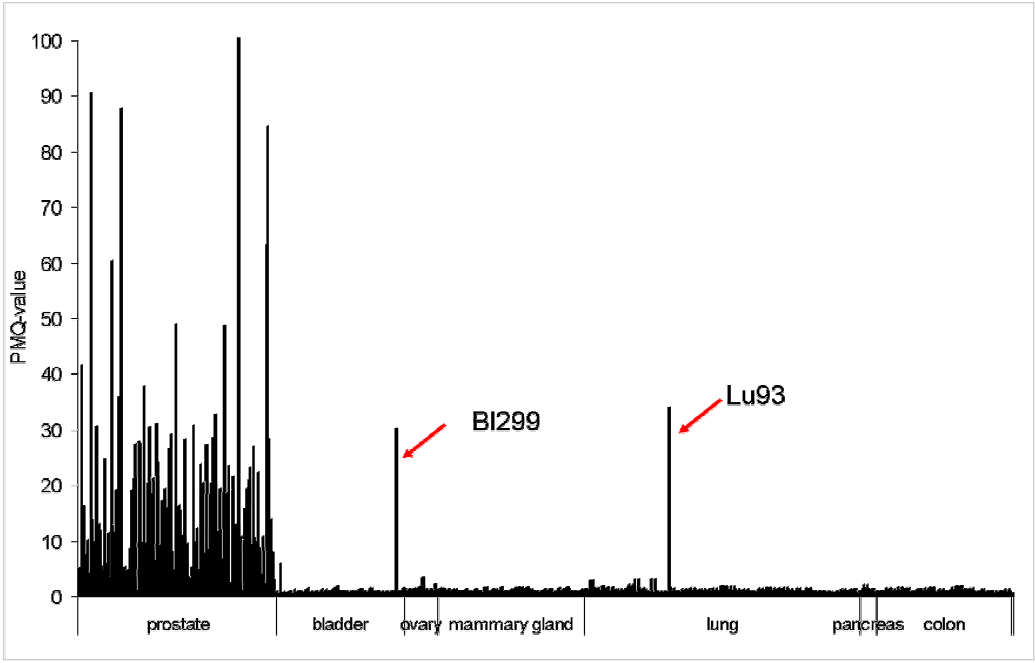

Fig. 19 Expression of TRPM8 in Affymetrix GeneChip experiments of 7 human tissues. Hybridization experiments were performed with 123 prostate -, 21 ovary -, 90 mammary gland -, 78 bladder -, 102 colon -, 11 pancreas - and 172 lung –specimens of normal and cancer tissue.

These results were quite unexpected as dot blot and Northern blot experiments showed exclusive expression of TRPM8 in the prostate. The bladder and the lung cancer sample stoud out very clearly from the other samples. Looking closer at the bladder cancer patient, this sample did not only express TRPM8, but also Kallikrein (PSA), Folathydrolase1 (PSMA) and the Acidic Phosphatase Prostate (ACPP) all of which are prostate specific markers (Fig. 20). None of the other 71 bladder samples did express any of these genes. In general these 4 genes were expressed conjointly in nearly 100% of all prostate samples, but very rarely (and never all 4 gene together) in any non-prostatic tissue tested (data not shown). As it was most unlikely that this patient really expressed prostate specific genes, the pathologist at the University of Regensburg (from where the tissue came from) was asked to diagnose this sample again. In response, she told us that this patient was indeed a bladder cancer patient, but that some prostate glands had infiltrated into the bladder. Unfortunately these glands were also microdissected and thus amplified and hybridized on the chip. Consequently this sample was taken out of further analysis of the bladder-experiments at metaGen. The most important aspect of this feature was not the fact that the sample was no pure bladder sample, but the confidence it provided regarding the data gained from the microarray experiments.

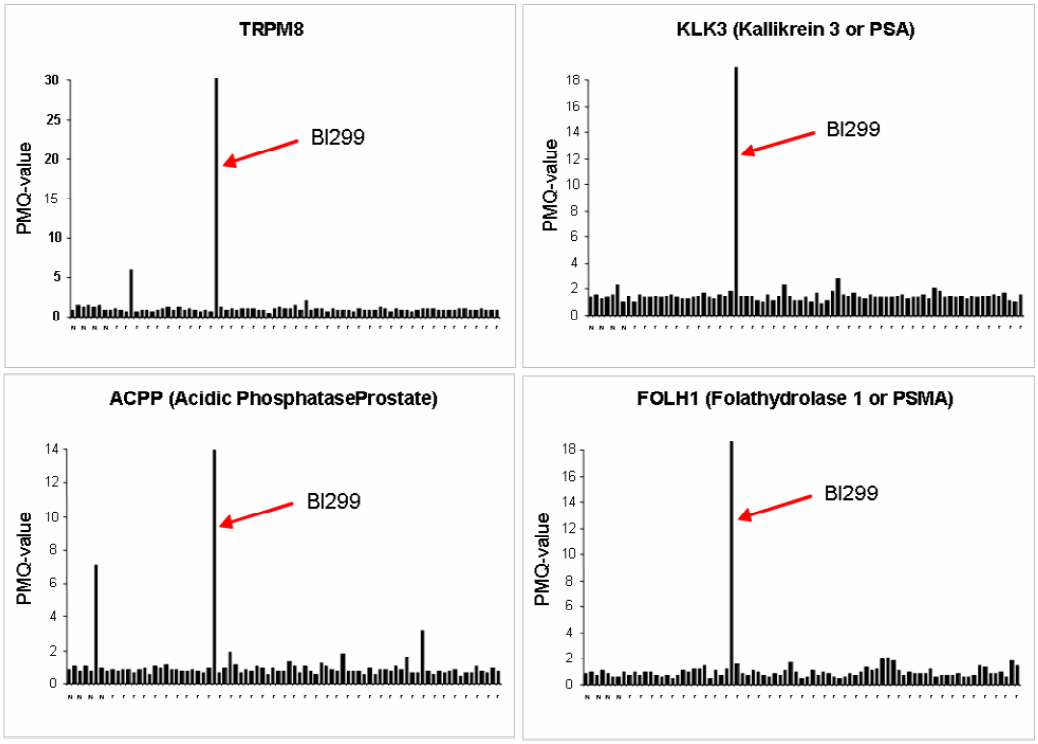

Fig. 20 Affymetrix GeneChip analysis of prostate specific genes in bladder cancer patients. Expression values are shown as PMQ for TRPM8, KLK3, FLOH1 and ACCP.

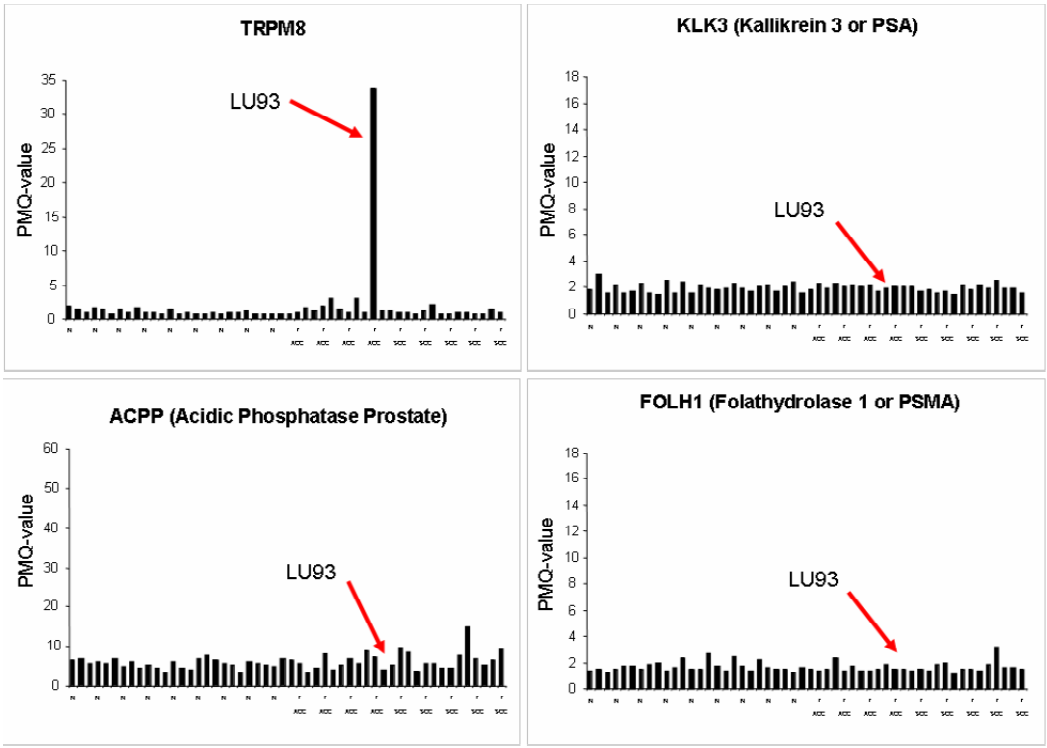

Fig. 21 GeneChip analysis of prostate specific genes in lung cancer patients. Expression values are shown as PMQ for TRPM8, KLK3, FLOH1 and ACCP.

Strikingly, in lung cancer patients TRPM8 was the only prostate specific gene expressed (Fig. 21). Neither Kallikrein nor Folathydrolase1 nor acidic phosphatase were expressed in this sample. A contamination with prostatic tissue or a prostatic metastasis in the lung could thus be excluded. In order to find an answer to this result the patients data sheets were checked in collaboration with the pathologist and an interesting observation could be made: Lu93 was the only patient run on microarray chips which had a fraction of 10% neuroendocrine tumor cells.

2.7 TRPM8 EXPRESSION IN NEUROENDOCRINE TUMORS OF THE LUNG

Real-Time PCR was performed to evaluate the assumed expression of TRPM8 in the patient with a 10% neuroendocrine tumor of the lung (Lu93). Additionally, a 100% neuroendocrine tumor from the lung and a lung adenocarcinoma were analyzed. The mRNA of normal and tumor material of these patients was isolated and quantitative PCR was performed. Results shown in Fig. 22 indicate that TRPM8 is highly overexpressed in both the 10% and the 100% neuroendocrine tumors, but not in the andenocarcinoma of the lung.

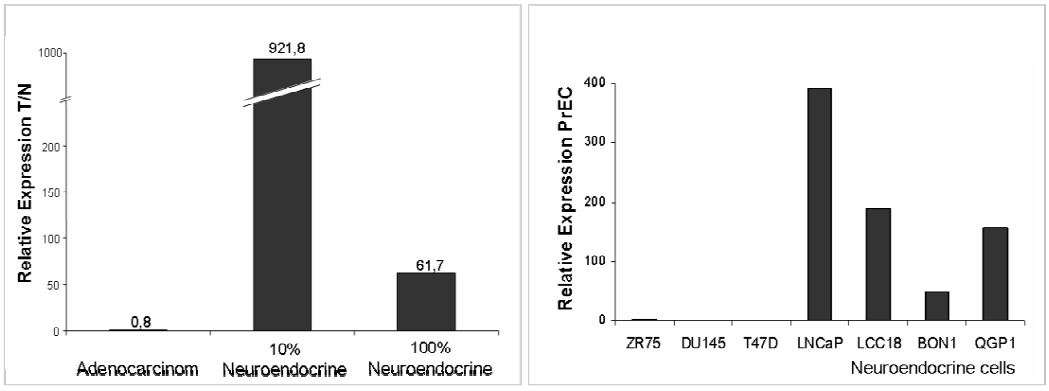

Fig. 22 Strong overexpression of TRPM8 in neuroendocrine tumors. Real-Time PCR of TRPM8 shown as relative expressions to corresponding normal tissue of each patient. **A)** Pure adenocarcinoma of the lung (panel 1), a lung adenocarcinoma with 10% of neuroendocrine cells (panel 2) and a 100% neuroendocrine tumor also located in the lung (panel 3). **B)** Expression of TRPM8 in neuroendocrine cell lines. RT-PCR results are shown as relative expression levels to the normal prostate epithelial cell line PrEC.

2.8 GENOMIC STRUCTURE OF TRPM8

TRPM8 is located on chromosome 2q37.2 TRPM8 distributed over 100kb at the very end of chromosome 2. The gene consists of 26 exons resulting in 5641 base pairs (Fig. 23). The open reading frame (ORF) has 3312 basepairs resulting in an ORF of 1104 amino acids. The ion pore of TRPM8 consists of 6 transmembrane spanning domains located between exon 16 and 20. Both N and C-termini are located in the cytoplasm.

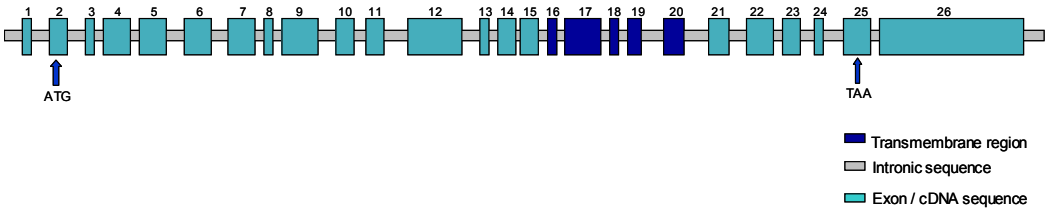

Fig. 23 Genomic structure of TRPM8 on Chromosome 2q37.2.

2.9 IDENTIFICATION OF TRPM8 SPLICE VARIANTS

TRPM8 splice variants could be identified while cloning the TRPM8 full length gene. The TRPM8 gene was amplified from a normal prostate mRNA pool (Ambion, Huntington, UK). A polyT-T7 primer was used in the reverse transcription RT-reaction which was followed by a PCR reaction using TRPM8 specific primer which bound to the first and last exon of TRPM8. It was interesting to observe that after cloning and sequencing of the constructs a short variant of TRPM8 could be identified. Exons 1-6 of this variant were identical to TRPM8, but exon 6 was elongated by 245 bp ending with a polyadenylation signal (SV 6b). Cloning of the product was possible as the primer used for the cDNA synthesis functioned as the reverse primer in the PCR reaction. This finding was in accordance with the results gained in the Northern blot experiments where two other isoforms of TRPM8 could be detected, although splice variant 6b was smaller than the shortest fragment (4.1kb) seen in the Northern blot experiments (Fig. 11).

In the course of performing genome database search using public[5] and proprietary (Incyte Genomics, Palo Alto, CA, USA) cDNA libraries 5 additional alternative transcripts of TRPM8 (Fig. 24) were identified. Using PCR-techniques it was possible to elongate most of the isoforms. The splice variant 6b of the TRPM8 has the SEQ ID NO 1. The splice variant 4a_4b has the SEQ ID NO: 2. It contains of at least 5 exons. Exon 2x is a separate exon (3'end is incomplete) and is located several hundred bases in front of exon 3 of trp-p8. Exon 3 is transcribed as in trp-p8 but exon 4 starts 46 bases earlier compared to trp-p8. The sequence continues from exon 5 to 6a and ends with 6b. Splice variant 16b of TRPM8 has the SEQ ID NO 3. It contains of 16 exons. Exons 1-15 are identical with TRPM8. Exon 16 has an extention of 104 basepairs immediately beginning after exon 16 and ending in a poly-A signal.

[5] http://www.ncbi.nlm.nih.gov/dbEST/

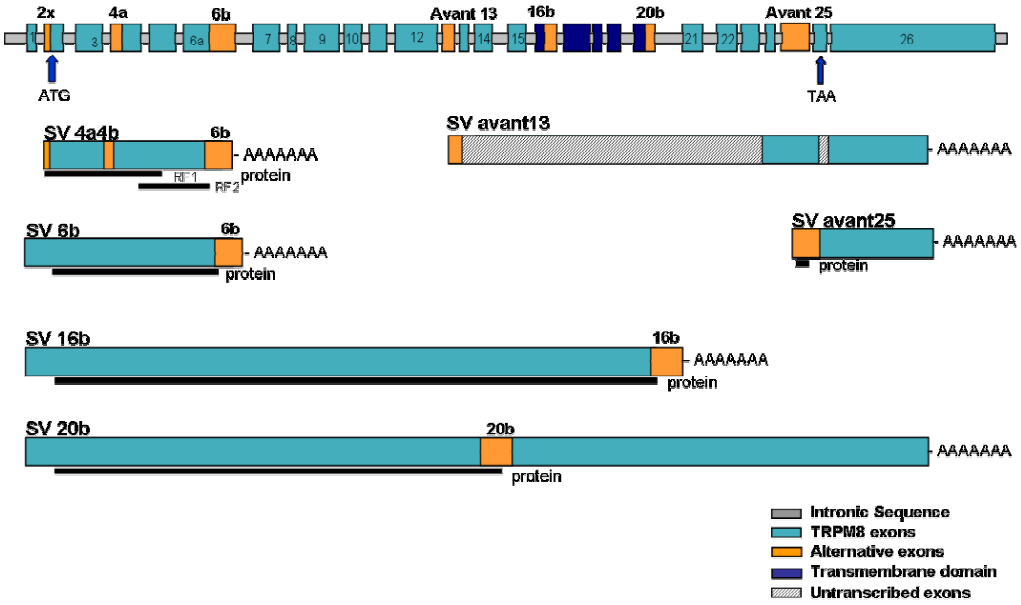

Fig. 24 Six splice variants of TRPM8. Alternative exons of TRPM8 are indicated as black boxes. Exons of TRPM8 are shown in grey and regions which are not transcribed are shown in stripy.

Splice variant 20b has the SEQ ID NO: 4. The sequence contains of 26 exons. Exons 1-19 and 21-25 are identical to TRPM8. Distinct from TRPM8 splice variant 20b contains an elongated exon 20 with an addition of 127 basepairs immediately beginning after exon 20; exon 26 is truncated: The poly-A signal starts already at basepair 1136. The splice variant avant25 of TRPM8 has the SEQ ID NO: 5. This sequence contains of at least 3 exons with an unknown 3' end. Avant25 contains an exon of 570 basepairs not found in TRPM8. This exon extends into in exon 25 and 26 but exon 26 is truncated as in splice variant 20b with a poly-A signal beginning at basepair 1136. The splice variant avant13 has the SEQ ID NO: 6. The sequence contains of at least 6 exons with an unknown 3' end. Avant13 has an exon of 272 basepairs not found in TRPM8. This exon extends into exon 21 but only 20 bases of the 3'end of this exon are within the transcript. The last 4 exons are identical to TRPM8 with a different splice pattern (exons 22, 23, 24 and 26, but exon 26 is truncated with a poly-A signal beginning at basepair 654).

Though it was possible to identify more splice variants, but as they were less abundant and less differentially expressed than those 5 they will not be further discussed here.

The structure of TRPM8 isoforms 16b and 20b is shown in Fig. 25. 16b has one transmembrane spanning domain which results in an extracellular C-terminus. Isoform 20b consists of the same transmembrane spanning domain as TRPM8 but the C-Terminus ends a few amino acids after the last transmembrane spanning domain.

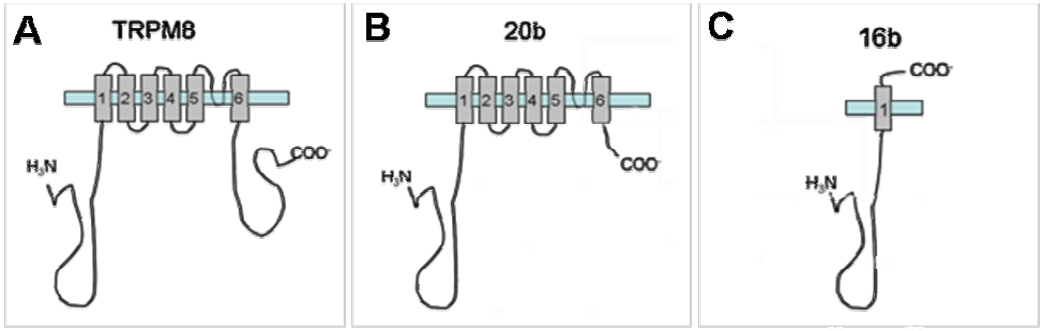

Fig. 25 schematic structure of TRPM8 its isoforms 20b and 16b. Number boxes (grey) indicate tranmembrane spanning domains. The blue horizontal beam represents the cell membrane.

2.9.1 Identification of the TRPM8-regulatory-RNA

On the basis of *in silico* analysis of different ESTs in the region of TRPM8 it was possible to identify a gene which is positioned on the opposite strand of the TRPM8 gene on chromosome 2q37.2. (SEQ ID NO: 6) Exon 1 of this RNA lies in intronic regions between exon 12 and 11 of TRPM8; Exon 2 lies in front, over, and behind exon 11. Exon 3 is located between exon 8 and 7 of the TRPM8 gene (Fig. 26). It was named TRPM8-Regulatory-RNA, because it may bind to TRPM8 or its splice variants and thus alter the expression of these genes. Additionally, binding of the mRNA may cause destabilization through activation of mRNA degradation mechanisms or stabilization of the mRNA altering in an elongated translation.

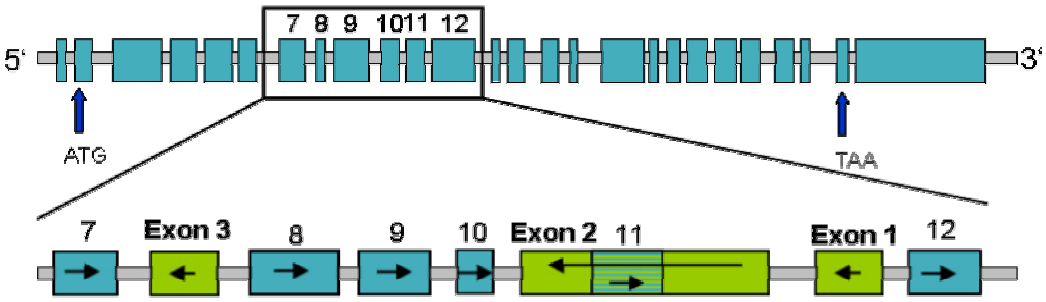

Fig. 26 Genomic localization of the human TRPM8-Regulatory-RNA. The exons of TRPM8 are marked in grey. Black boxes indicate exons of TRPM8 regulatory RNA. Arrows indicate the direction of transcription.

2.9.2 Expression of TRPM8 splice variants in prostate tumors

Real time PCR of the 5 splice variants and the regulatory RNA was performed in order to find out whether these isoforms are as differentially expressed as TRPM8 itself. PCR was performed on samples from prostate cancer tissues used for gene chip experiments and on some additional samples. Tab. 3 shows the results. The splice variants 16b, 20b and avant25 were overexpressed in prostate tumors to 65%, 67% and 60%, respectively, altering an even higher differentially expression than TRPM8 itself. The TRPM8-Regulatory-RNA was overexpressed

in 80% of the prostate patients which was the highest differential expression seen. Additionally, relative expression values between a corresponding normal and cancer sample of the isoforms were significantly higher than those of TRPM8 (data not shown). But absolute expression levels of the slice variants were generally lower.

Tab. 3 Summary of quantitative RT-PCR of splice variants of TRPM8. Expression values were normalized to ß-actin and relative expression was calculated using the ΔΔ CT method.

Splice Variante	Number of Patient (T/N > 4)	% of Patients (T/N > 4)	Number of Patient (N/T > 4)	% of Patients (N/T > 4)
TRPM8	23 von 42	**54 %**	1 von 42	2%
6b	7 von 14	**50%**	1 von 14	7%
16b	28 von 43	**65%**	0 von 43	0
20b	28 von 42	**67%**	2 von 42	5%
avant25	9 von 15	**60%**	0 von 15	0
avant13	7 von 14	**50%**	0 von 14	0
Regulatory RNA	12 von 15	**80%**	1 von 15	7%

2.9.3 Characterization of TRPM8 Splice variant 16b

The splice variant 16b was chosen for a detailed analysis because a) it is one of the highest differentials expressed splice variants in prostate cancer patients seen in RT-PCR experiments (Tab. 3) and b) most importantly, the N-terminus is probably located in the extracellular space. This extracellular localization makes it an ideal target for the development of a therapeutic antibody, even better than TRPM8 itself as it has a larger epitope for the production of an antibody.

In order to determine the expression profile of splice variant 16b, Northern and dot blot experiments with the ^{32}P-labeled 16b-specific probe were performed on the commercial Cancer Profiling Array (Clonech, Heidelberg, Germany) (Fig. 27).

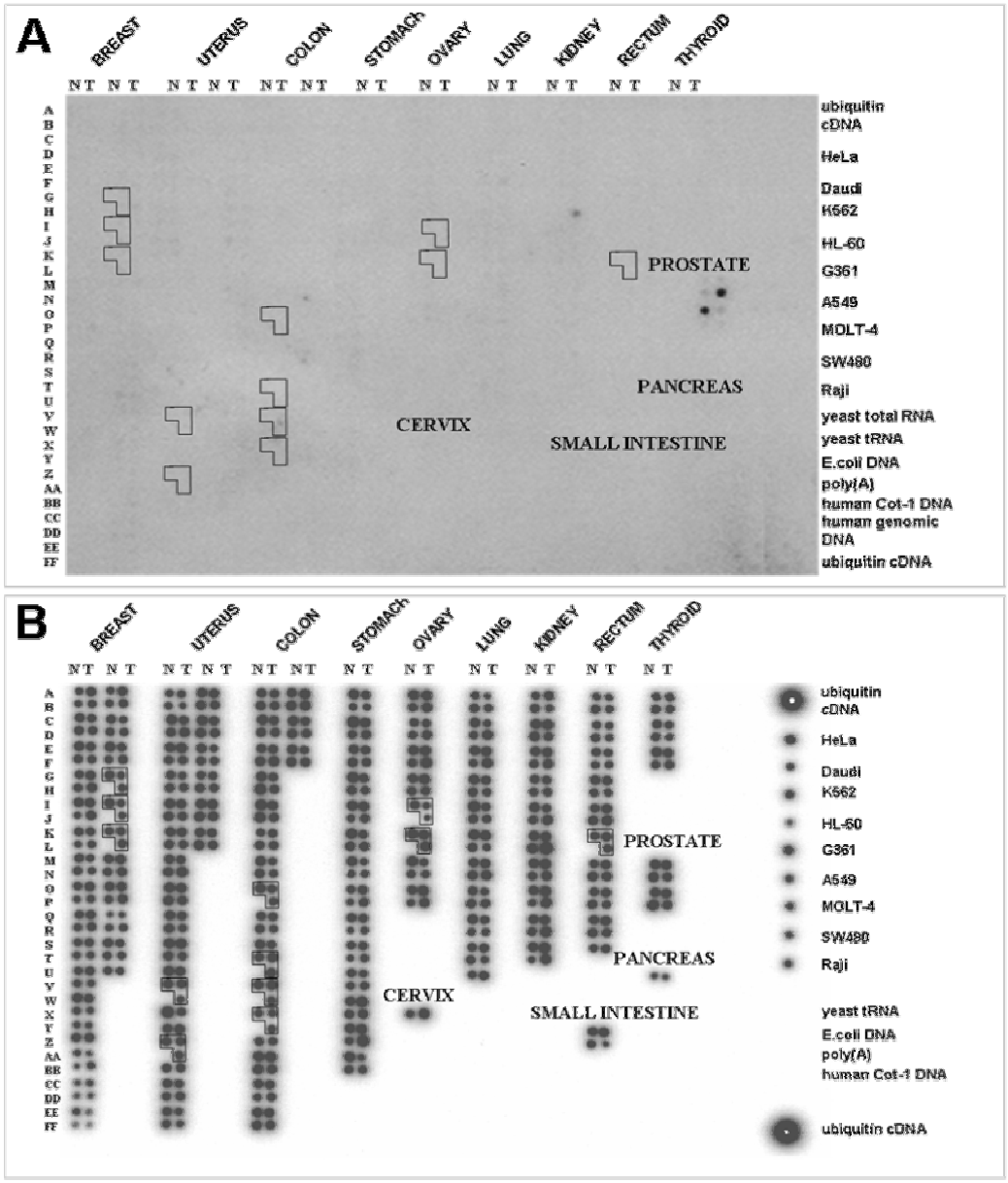

Fig. 27 Cancer Profiling Array representing 241 matched tumor and normal human tissues from 13 cancer entities and several cell lines. A) The 16b specific probe was 32P-labeled and hybridized to the membrane. **B)** The Ubiquitin specific probe was 32P-labeled and hybridized subsequently to the same blot.

Additionally dot blot experiments were carried out on a self spotted blot, to which cRNA from 48 tumor and corresponding normal prostate tissues were spotted to the membrane (Fig. 28). The cRNA spotted was derived from amplified cRNA used for gene chip experiments. Strikingly, the expression pattern of 16b is the same as for TRPM8. The self made dot blots reveal that 16b is overexpressed in 65% of all prostate tumors. These results show that SV 16b would be an even better target for the development of a therapeutic antibody than TRPM8.

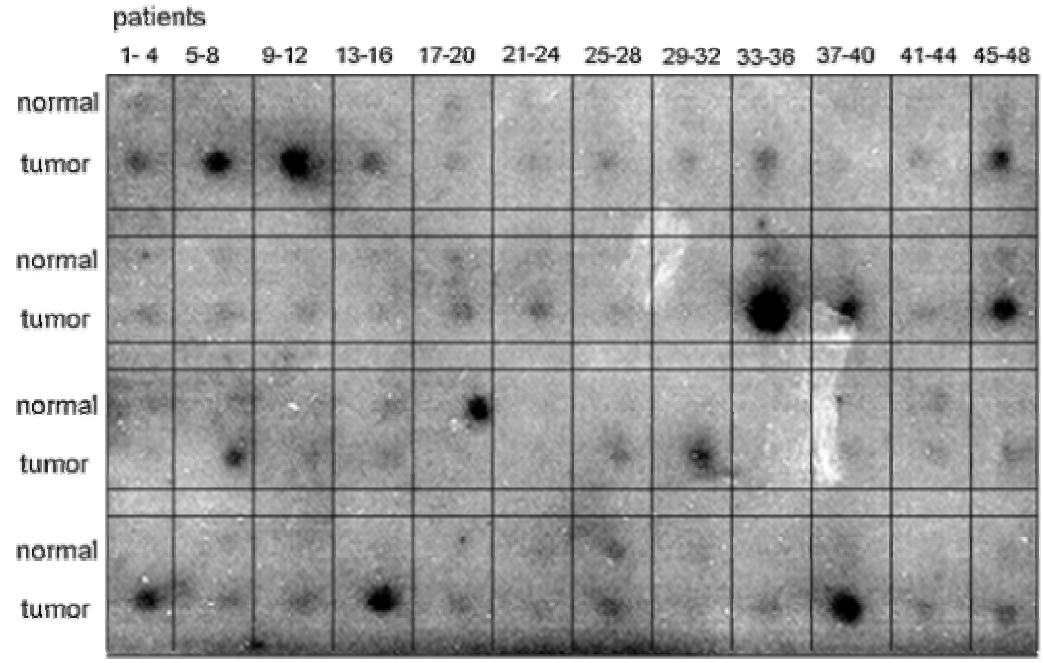

Fig. 28 Dot blot of matched prostate cancer and normal tissue hybridized with a SV 16b specific probe. cRNA of samples used for hybridization in Affymetrix microarray analysis were spotted to a nitrocellulose membrane a 16b specific probe was 32P-labeled and hybridized to the membrane.

2.9.4 Generation of HEK293 cell stable for 16b and TRPM8

HEK293 cells stable for TRPM8-pcDNA3.1-V5 were transfected with the 16b-pcDNA6-myc constructs for 24h. After antibiotic selection clones were checked for TRPM8 and 16b expression using V5- and myc-specific fluorescent antibodies in the fluorescence activated cell sorter (FACS) (Fig. 29). The percentage of cells which were positive for TRPM8 ranged from approximately 52% to 71% (Fig. 29 A and B). By contrast, cells positive for 16b exhibited only a percentage of 1,3 to 7,6% (Fig. 29 C and D). For control purpose these constructs were checked in Western Blot experiments for the expression of SV 16b (88kD). These experiments showed a strong expression of 16b in all of the six clones tested (Fig. 30).

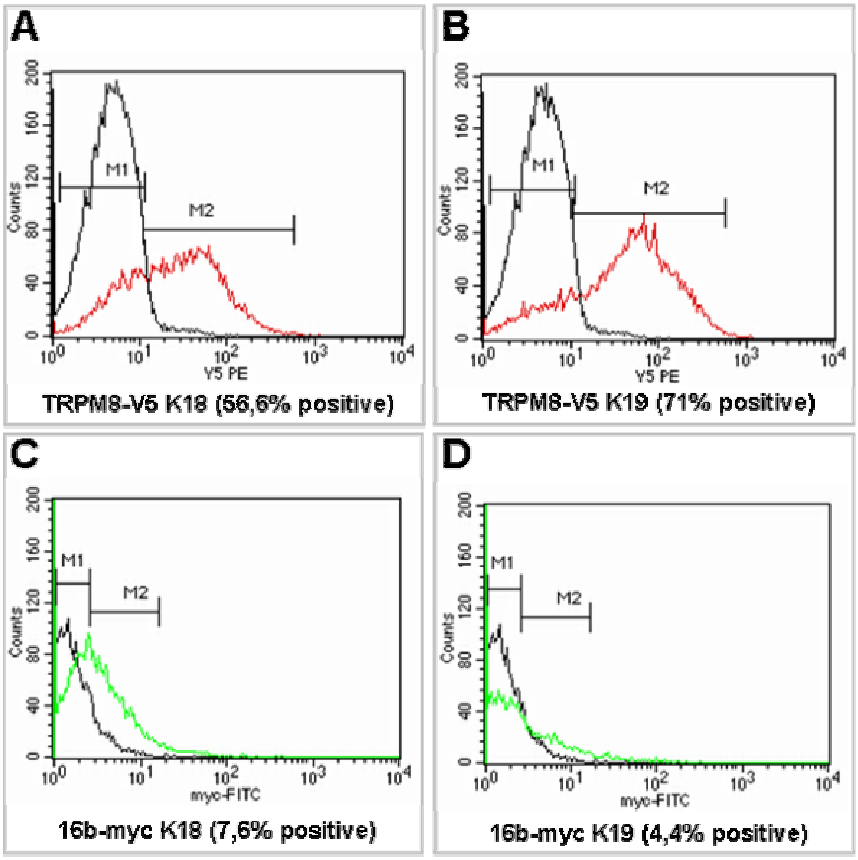

Fig. 29 FACS analysis of TRPM8 and 16b stable transfected cells. HEK293 cells stable transfected for TRPM8 and 16b were double stained for V5- and myc-epitope with FITC and PE labeled antibodies respectively. A) and B) show TRPM8-V5 expression of clones 18 and 19 stained with the anti-V5-PE antibody. C) and D) show the same clones, this time stained with anti-myc FITC antibody. M1 represents the mock clone (pcDNA3-1-V5-TOPO (A+B) and pcDNA6-myc-his (C+D). M2 gates the positively stained cells for TRPM8 (A+B) and 16b (C+D).

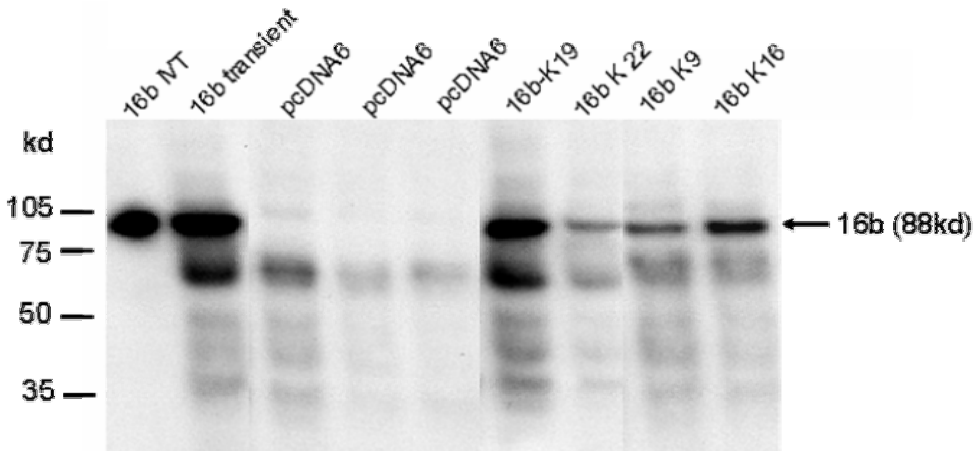

Fig. 30 Western Blot of TRPM8-HEK293 cells stable transfected with 16b. Detection was performed with an anti-myc antibody. Lane 1 shows the 16b-protein of an *in vitro* translation reaction; lane 2 the transient transfected protein; lane 3-5 the empty vector of pcDNA6-myc-his (mock), lane 6-9 shows the 16b stable transfected TRPM8-HEK293 clones (Clone 19, 22, 9 and 16).

2.9.5 Cellular localization of TRPM8 and SV16b in HEK293 cells

Cellular localization of TRPM8 and its splice variants were analyzed by cloning TRPM8 and 16b into the pcDNA3.1-V5-TOPO and pcDNA6-myc-his vector, respectively. HEK293 cells were used for the genration of stable cell lines expressing TRPM8. SV 16b was transiently transfected into these cells and also into wild type cells for 24 hours, prior fixation and staining with fluorescent antibodies for V5 and myc. Subcellular distribution of TRPM8 and 16b was detected by confocal fluorescence microscopy (Leica Microsystems, Solms, Germany) Fig. 31.

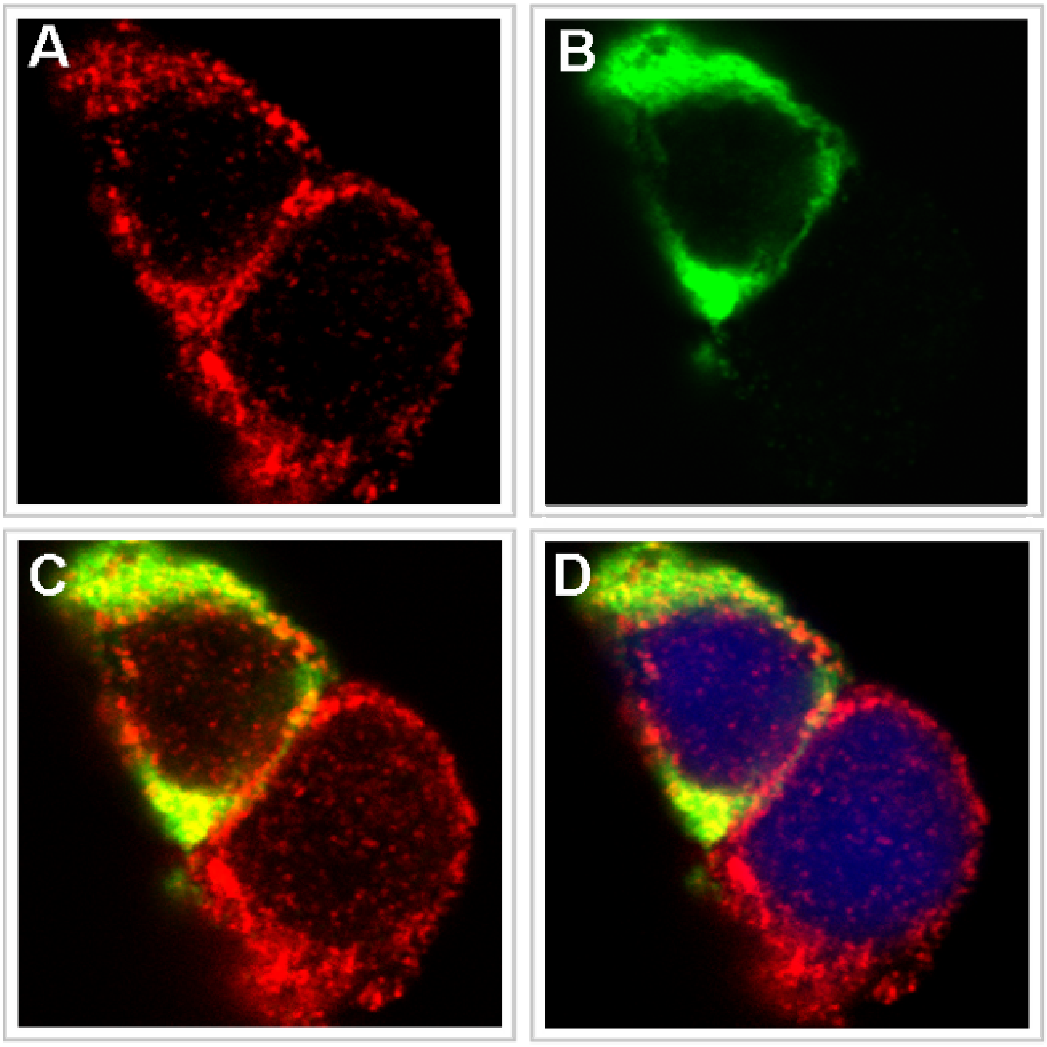

Fig. 31 Co-expression and cellular localization of TRPM8 and SV 16b in HEK293 cells. A) Expression of TRPM8-V5 (red), **B)** Expression of 16b-myc (green), **C)** Overlay of pictures **(A)** and **(B)**. D) Overlay of pictures (A) and (B) plus DAPI staining (blue).

TRPM8 expression could be seen within the cytosol and in the membrane of intracellular compartments, predominantly in the endoplamatic reticulum. The expression exhibited a spotty cluster, which has already been shown for other TRP channels as for example TRPC3 [Hofmann, 99] (Fig. 31 A). By contrast

16b distributed homogenously within the cytoplasm which led to the conclusion that 16b is a soluble protein (Fig. 31 B). In order to examine whether 16b interacts in any form with TRPM8 in vitro, co-transfection of SV 16b in to HEK293 cells stabel transfected for TRPM8 were performed. As shown in Fig. 31 C and D 16b did not alter the localization of TRPM8.

2.9.6 Influence of SV 16b on the activation of TRPM8 by icilin

In order to examine whether the splice variant 16b had any influence on the activation of TRPM8 by icilin different HEK293 clones expressing TRPM8 and 16b stably, were exposed to 50 µM of Icilin. It could be shown that 16b reduces generally the calcium influx in HEK293 cells. (Fig. 32 D - F), but the results obtained were very unstable. For an unknown reason the empty vector control showed also some inhibiting function (repeated experiments). These findings were supported by FLIPR calcium assay experiments. Fig. 33 shows the calcium influx which was induced by 1 µM of Icilin in TRPM8 and 16b transfected HEK293 cells. Again, wild type HEK293 cells show no increase in intracellular calcium when exposed to Icilin. Only when these cells were transfected with TRPM8 a calcium flux could be measured. When cells were co-transfected with TRPM8 and 16b the influx in calcium was quite noticeable reduced, but some clones of 16b transfected cells show an increase in calcium flux (i.e. Clone 19). Additionally, the empty vector (ev) control showed also reduced flux of Ca^{2+}.

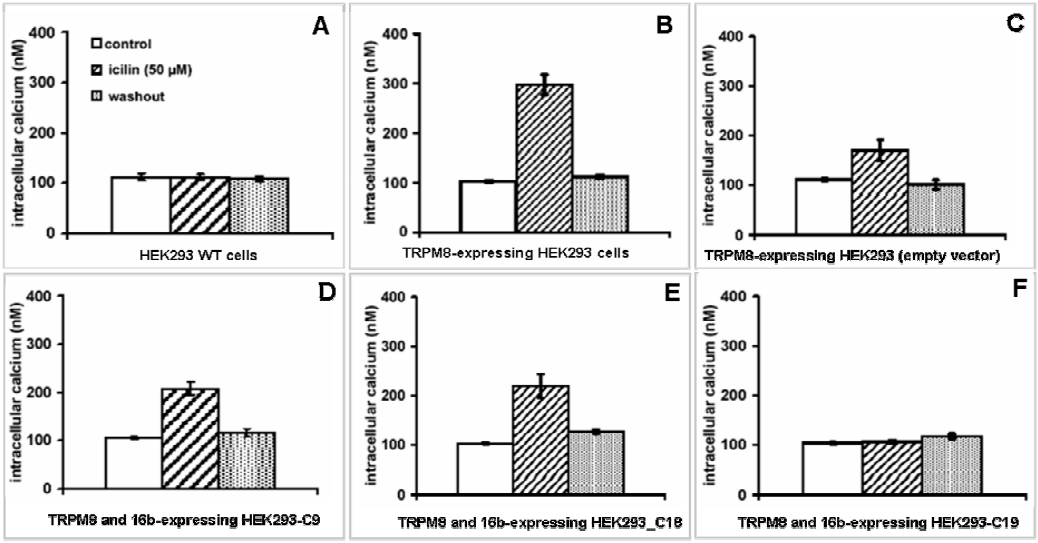

Fig. 32 Analysis of calcium influx of HEK293 cells co-expressing TRPM8 and 16b using Fura-2. Representative measurement data were taken from calcium experiments of transfected and nontransfected HEK293 cells loaded with Fura-2. WT = wild type.

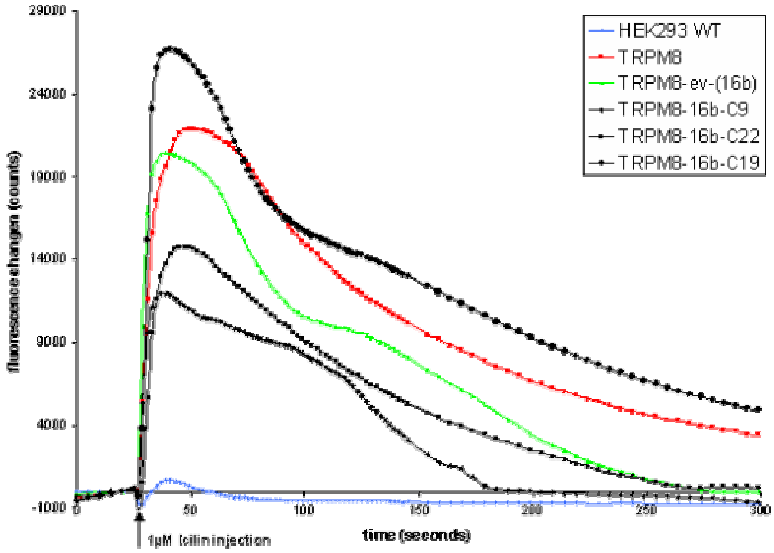

Fig. 33 Calcium flux induced by 1 µM Icilin in TRPM8 and 16b transfected HEK293 cells. Free calcium was measured using the FLIPR calcium assay kit and is presented as a change in fluorescence versus time.

2.9.7 Aberrant splicing of SV 16b

In RT-PCR experiments using TRPM8 and 16b specific primers, it was further possible to identify even more splice variants named 16b -1 to 16b - 4 by RT-PCR using primer specific for TRPM8 (forward primer) and 16b (reverse primer) (Fig. 34). Sequencing of the PCR products revealed the exon scipping shown in Fig. 34 B.

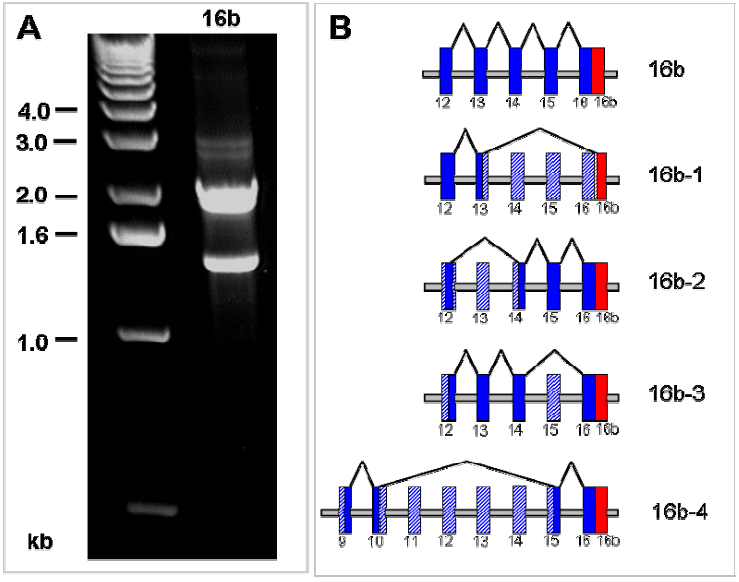

Fig. 34 Aberrant splicing of splice variant 16b. A) Agarose gel of a RT-PCR with 5' TRPM8 and 3' 16b specific primers. **B)** Blue indicates transcribed exons, shaded blue and red indicates untranscribed exons and red marks the alternative exon 16b.

2.10 PROMOTORANALYSIS OF THE TRPM8 GENE

In the first part of this study it was demonstrated that TRPM8 is an extremely tissue specific gene expressed exclusively in the prostate and neuroencorine tumors. Out of this characteristic arose the question, whether this specificity could be used not only for a small-molecule or antibody based therapy but also for gene therapy. The idea was to clone the TRPM8 promoter in front of the sequence of a certain toxin, such as diphteria toxin A into a viral vector used for gene therapy (Fig. 35) (Li, Diphteria Toxin, cancer research 2002). Expression of the toxin would be restricted to the prostate as the transcription of the toxin would be under the control of the tissue specific TRPM8 promoter. Following that approach several questions need to be answered. 1. How does the TRPM8 promoter look like and which transcription factor (TF) binding sites are present? 2. Is the promoter responsible for the tissue specific expression of TRPM8? 3. And if, is it possible to narrow down a specific part (TF- binding site) responsible for transcriptional activation or repression? 4. As it has been shown that TRPM8 is expressed under the control of androgens, are androgen responsive elements (ARE's) present? All of these questions will be addressed in the next chapters.

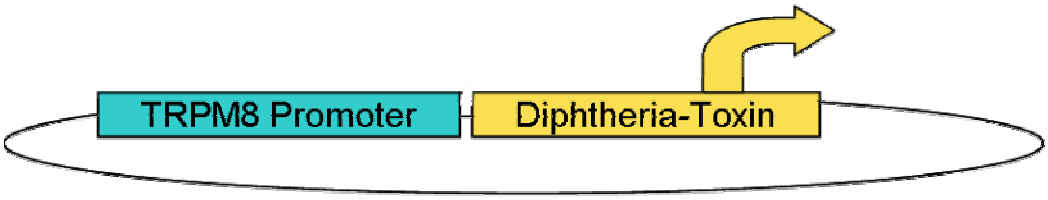

Fig. 35 Gene therapy approach using the TRPM8 promoter as the prostate specific transcriptional regulator of the Diphteria-Toxin-A expression.

2.10.1 Characterization of the TRPM8 Promoter

TRPM8 is a very tissue specific gene of which the expression is restricted to the prostate and neuroencorine tumors. One goal of this study was to identify and characterize the promoter of TRPM8. By blasting the cDNA sequence of TRPM8 gene against the public HTGS (High Throughput Genomic Sequences) database[6] a BAC AC005538 was identified which covered the whole genomic region of TRPM8 including 100 MB of the 5' regions. Fig. 36 shows the 1.9 kb sequence of the TRPM8 promoter including the short 5' UTR as well as the transcription start site and the first exon of TRPM8. That fragment was subjected to *in silico* analysis for potential transcription factor binding sites using the commercially available software MatInspector[7] [Quandt, 95].

[6] http://www.ncbi.nlm.nih.gov/HTGS/

[7] http://www.gsf.de/biodv/matinspector.html

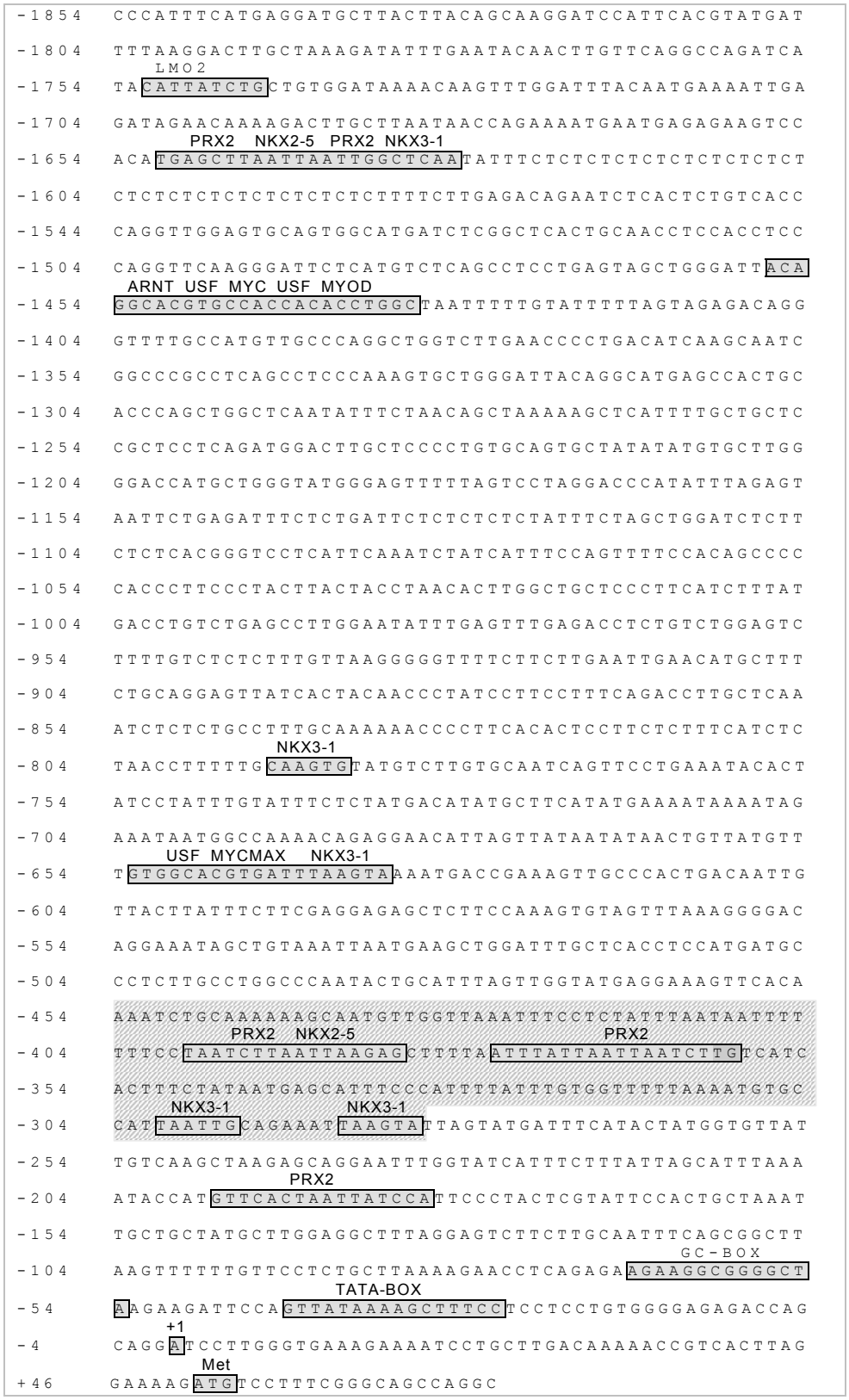

Fig. 36 Sequence of the promoter region of the human TRPM8 gene on 2q37.2. Grey boxes indicate putative transcription factor binding sites. +1 shows is the transcription start site. Met = methionine, shows the translation start site.

This software utilizes the TRANSFAC[8] library, which emphasis on sequences with experimentally verified binding capacity. Tab. 4 lists the transcription factors with the highest prediction values. The score for the core sequences listed was in all cases 1.0 which means that the 4-8 basepairs (capital letters) matched 100% to the core sequence of the transcription factor from the database. The matrix similarity comparing the given sequence of the flanking region of the core with the sequence form the database were between 0.90 and 0.99 indicating that even the matrix similarity shows a high identity to known *cis*-elements (values > 0.8 were designated as good). For the transcription factor NKX3-1 the matrix value was left out as the identification of this transcription factor binding site was based on an own literature [Steadman, 00]. Steadman identified in gel shift experiments that these hexamers strongly bind NKX3-1. The average frequenz/1kb of these sequences was calculated by blasting each hexamer against a 5 MB sequence of Chromosome 21 which should represent a general distribution of the sequence within the whole genome.

Tab. 4 Transcription factor binding sites in the TRPM8 promoter. HGNC stands for the official name given by the "HUGO Gene Nomenclature Committee". The average frequency/1kb gives an expectation value of matches per 1kb of genomic DNA as given in TRANSFAC and * as blasted against a 200kb region of chromosome 21. Core similarity shows the similarity of the core sequence (usually between 4-6 base pairs) to the database sequences (capital letters). Matrix similarity shows the similarity of a sequence flanking the core region to the databases sequences (lower case letters). A perfect match of a matrix gets 1.0 a "good" match to the matrix usually has a similarity of > 0.80. Both calculation algorithms of the core and matrix are described in Quant et al. [Quandt, 95].

HGNC	*Position*	*Strand*	*Core Similarity*	*Matrix Similarity*	*Average Frequency / 1kb*	*Sequence*
LMO2	-1752	(-)	1,00	0,97	2,02	caGATAatg
PRX2	-1651	(+)	1,00	0,97	0,67	tgagcttaATTAattg
NKX2-5	-1647	(+)	1,00	0,97	0,65	ctTAATta
PRX2	-1645	(-)	1,00	0,99	0,67	ttgagccaATTAatta
NKX3-1	-1641	(+)	1,00	-	0,36*	TAATTG
ARNT	-1457	(-)	1,00	0,98	0,69	ggtggcaCGTGcctgt
USF1	-1456	(+)	1,00	0,99	1,67	caggCACGtgccac
MYC	-1455	(-)	1,00	0,97	1,64	tggcaCGTGcct
USF1	-1453	(+)	1,00	0,99	1,67	gCACGTgc
MYOD1	-1442	(+)	1,00	0,98	0,96	caCACCtggc
NKX3-1	-793	(+)	1,00	-	0,26*	CAAGTG
USF1	-653	(+)	1,00	0,99	1,67	gtggCACGtgattt
MYCMAX	-652	(-)	1,00	0,98	1,67	aatCACGtgcca
USF	-650	(+)	1,00	0,99	1,67	gCACGTga
NKX3-1	-640	(+)	1,00	-	0,31*	TAAGTA
PRX2	-399	(+)	1,00	0,97	0,67	taatcttaATTAagag
NKX2-5	-395	(+)	1,00	0,97	0,65	ctTAATta
PRX2	-377	(+)	1,00	0,99	0,67	atttattaATTAatct
NKX3-1	-301	(+)	1,00	-	0,36*	TAATTG
NKX3-1	-288	(+)	1,00	-	0,31*	TAAGTA
PRX2	-197	(+)	1,00	0,98	0,67	gttcactaATTAtcca
GC-Box	-67	(+)	1,00	0,90	2,12	agaaGGCGgggcta
TATA-BOX	-42	(+)	1,00	0,93	0,3	gtTATAAAAg

[8] http://www.gene-regulation.com/pub/databases.html#transfac

It was interestingly to note that the predominant transcription factor binding sites identified were from the family of homeobox genes. *Cis*-elements for the binding of PRX2 (paired related homeobox protein 2) and NKX3-1 was present 5 times each in the 1.9kb promoter sequence of TRPM8. This is a dramatic overrepresentation. The TRPM8 promoter contains a TATA-box and a GC-Box at positions -42 and -67, respectively. Transcription factor binding sites for NKX3-1 (5x), NKX2-5 (5x), USF1 (4x), MYCMAX, LMO2, MYC and ARNT were found in this fragment. Only those binding site were listed which have maximum similarity with described binding sites expressed in core and matrix similarity- values (Tab. 4).

2.10.2 Genomic structure of the human and mouse TRPM8 promoter

Genomic analysis of the promoter region of mouse and human TRPM8 revealed that the mouse orthologue misses the first exon of human TRPM8. But it alters 4 additional exons at the 5' end, which were so far not seen in humans (Fig. 37). A Dotter analysis of the 700 bp region 5' to the transcription start site of human TRPM8 to the mouse TRPM8 genomic region is shown in Fig. 38**.** It demonstrates the high homology between mouse and human of the 172-base pair conserved element. Additionally, it could be illustrated that the GC- and TATA-Box are also conserved between these species.

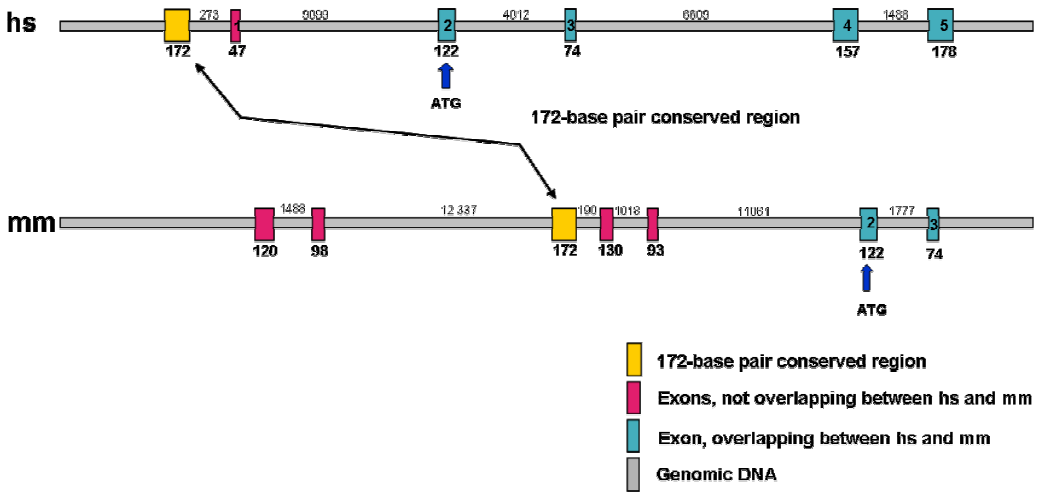

Fig. 37 Comparison of the genomic structure of the human and mouse TRPM8 promoter region.

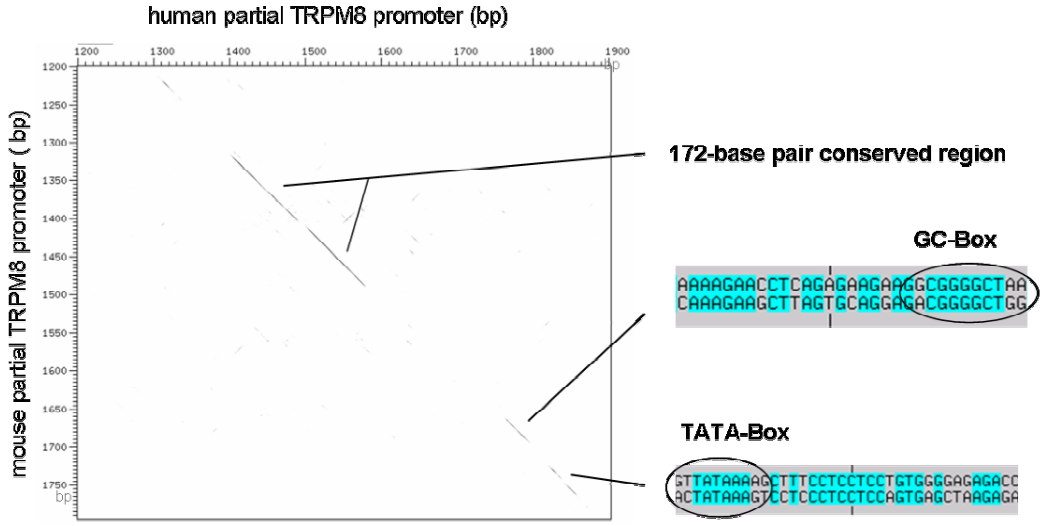

Fig. 38 Homology analysis of the TRPM8 promoter between human and mouse. The 700 bp 5` to the transcription start site of the human TRPM8 were dotted against the mouse genomic region of the TRPM8 gene. Grey lines indicate high homology between the sequence of human and mouse.

2.10.3 Transcription repression by a highly conserved promoter fragment

Alignment of the 1.9 fragment of the human TRPM8 promoter sequence with the mouse and rat orthologes revealed an overall identity between human and mouse of 56%. Strikingly a section of 172 bp showed a much higher concordance between the species than the overall sequence. Mouse and human display a similarity of 83% and rat and human of 82% (Fig. 36, Fig. 39). The first assumption, that this could be an alternative exon of TRPM8 was disapproved by repeated RT-PCR demonstrating that it was neither possible to amplify this fragment from any cDNA bank by itself nor with any known exon of TRPM8. To determine if the short part and the 1.9 sequence were able to induce transcription activation these two fragments were cloned each in front of the luciferase reporter vector pGL3-Basic. Different cell lines were transiently transfected with the constructs using PhRL-0 vector for transfection efficiency control. The 1.9 kb TRPM8 promoter showed a more than 13-fold transcriptional activation in DU145 cells (Fig. 40). In LNCaP, PC3 and HEK293 activation ranged from a 9-fold, over 7-fold to nearly 4 fold, respectively. Interestingly, the short element exhibited in all experiments a decrease in activation compared to the pGL3-Basic vector. This indicated that the conserved element might contain a repressor element (Fig. 40).

The observation that the 172bp and the 1.9kb fragment -although oppositional- both regulate expression identifies this region as promoter site of the human TRPM8 gene. Especially, because a TATA-box with a high significance could be identified at the expected 30-45 base pairs away from the start site. Within the conserved region transcription factor binding site for PRX2 (2x) NKX2-5 and NKX3-1 (2x) were identified. The responding transcription factors all belong to the group of proteins which are characterized by the presence of the homeobox which binds the DNA, indicating that these genes play a predominant role in the activation or repression of TRPM8.

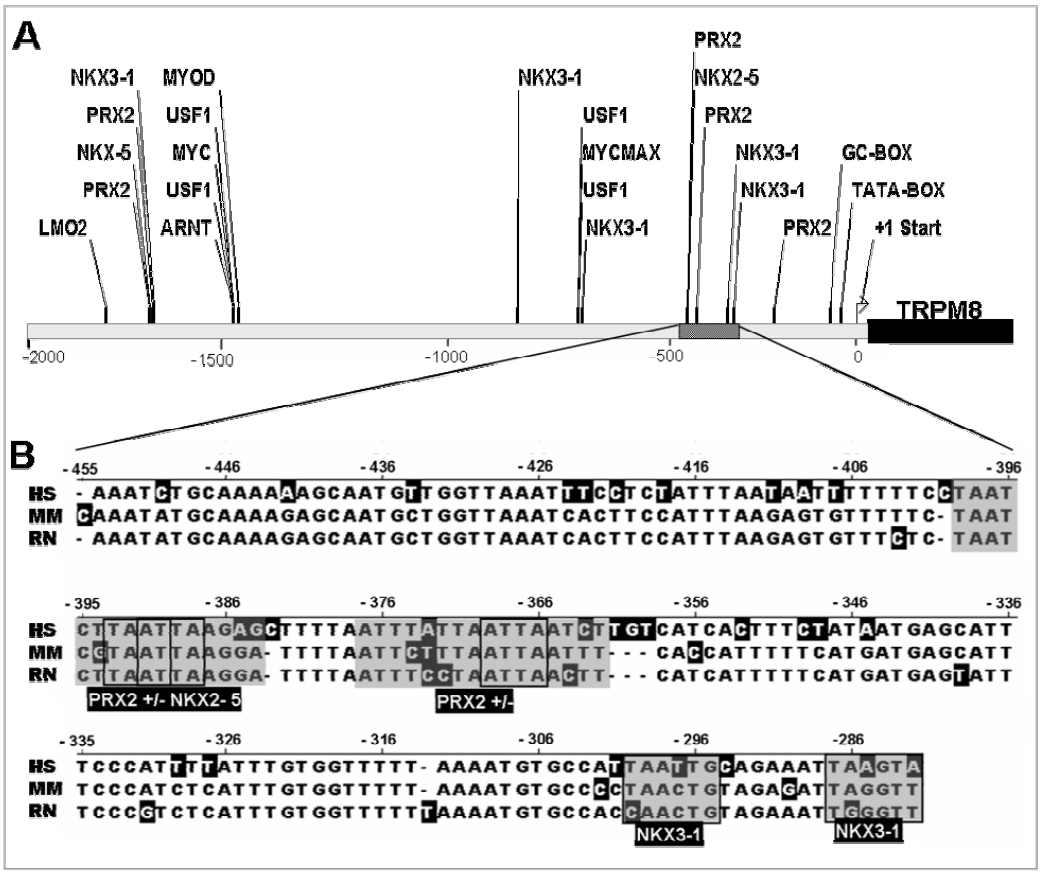

Fig. 39 The TRPM8 promoter revealing a highly conserved region. A) TRPM8 gene promoter. The transcription start is shown as +1, the black box indicates the TRPM8 protein; the grey box indicates a highly conserved sequence among species. **B)** Alignment of the human, mouse and rat highly conserved 172 bp region in the TRPM8 promoter. DNAs were aligned using CLUSTAL program. Grey boxes indicate the transcription factor binding sites with the 4-6 core base pairs framed in black.

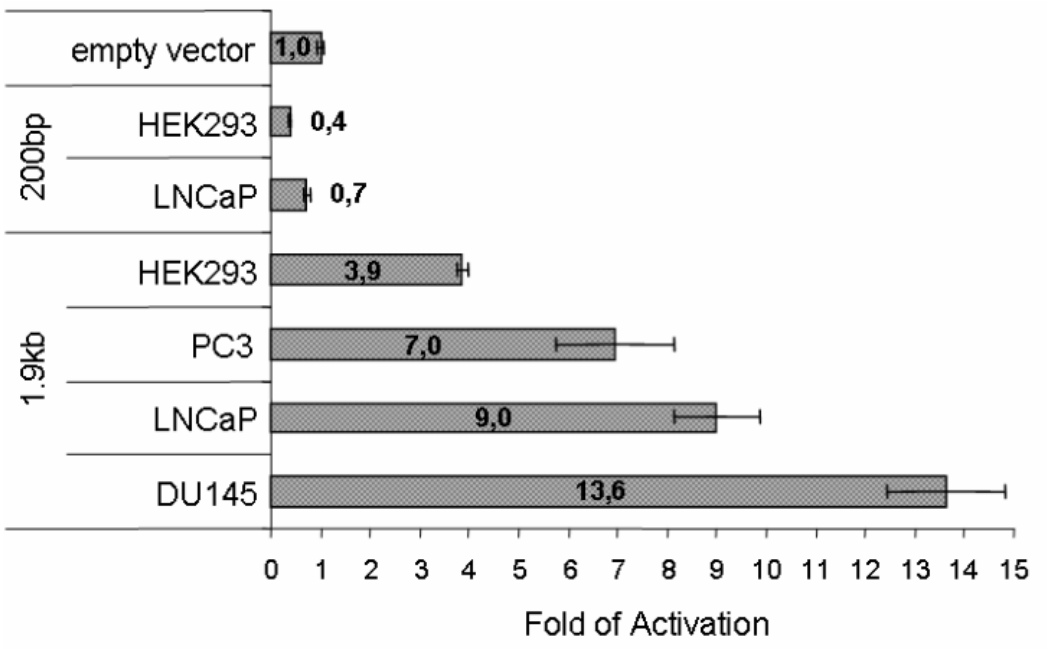

Fig. 40 Transcriptional activation of the TRPM8 promoter in different cell lines. HEK293, PC3, LNCaP and DU145 were transfected with the 1.9 kb-TRPM8 promoter fragment cloned in front of the luciferase reporter gene (1.9-kb-TRPM8-pGL3). Luciferase activity in the lysates was measured after 24 h. Data were normalized to the PhRL-0 which was used to normalize transfection efficiency. Data is shown relative to the pGL3-Basic vector.

2.10.4 Site-directed mutation in the 1.9kb promoter

The promoter of TRPM8 exhibits multiple transcription factor binding sites. In order to identify which transcription factor would alter the activity either through activation or repression 13 site-directed deletions of the core 6-8 base pairs of each potential TF-binding site were introduced into the 1.9kb-pGL3-reporter vector using site-directed mutagenesis (Fig. 41). LNCaP cells were transfected for 24 h with these constructs and luminescence was measured. All of the mutations led to a reduction of the activation potential. The strongest inhibition of activation (up to 80%) was achieved when the GC-Box or the TATA-Box were mutated. Neither all NKX3-1 nor PRX2 binding sites showed an agreeing repression pattern.

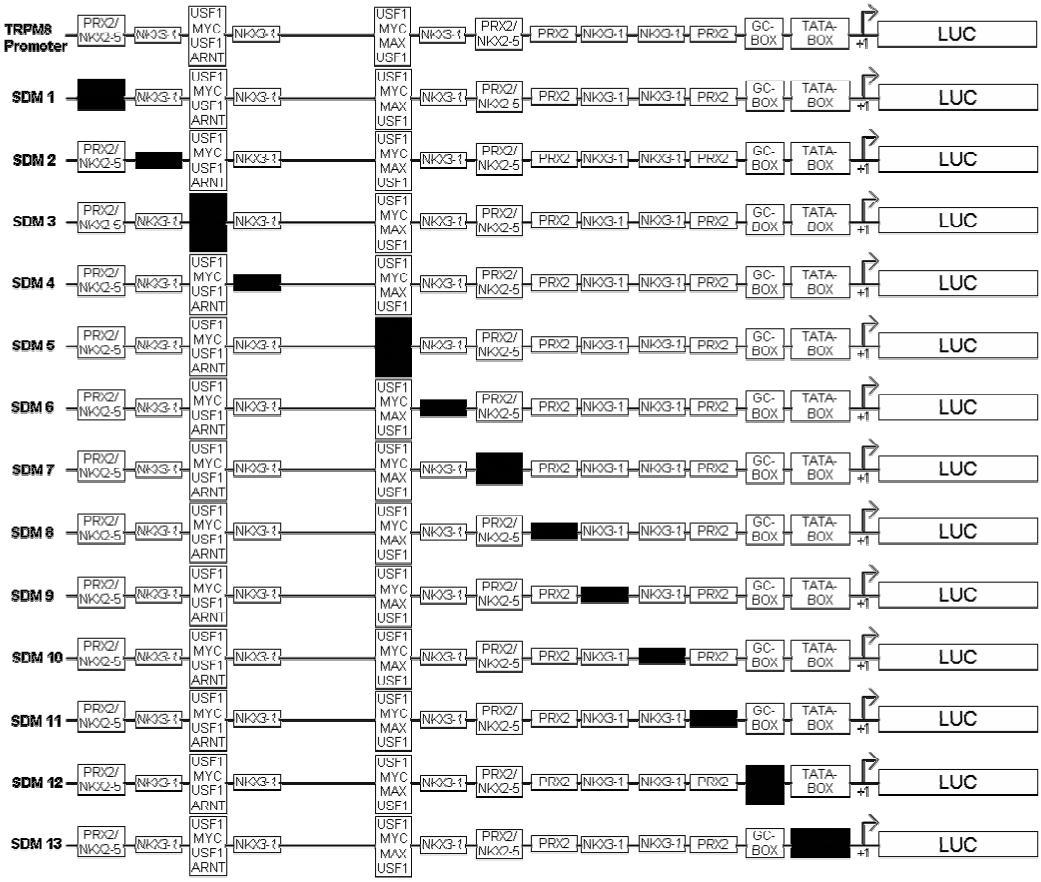

Fig. 41 Site directed mutated reporter gene constructs of the TRPM8 promoter cloned into the pGL3-basic reporter vector. The deletions in the constructs (SDM 1 to SDM 13) are marked in dark grey. In each mutation at least the core region of the transcription factor binding site was deleted, thus usually 6-8 bases were eliminated (see "Methods" for details).

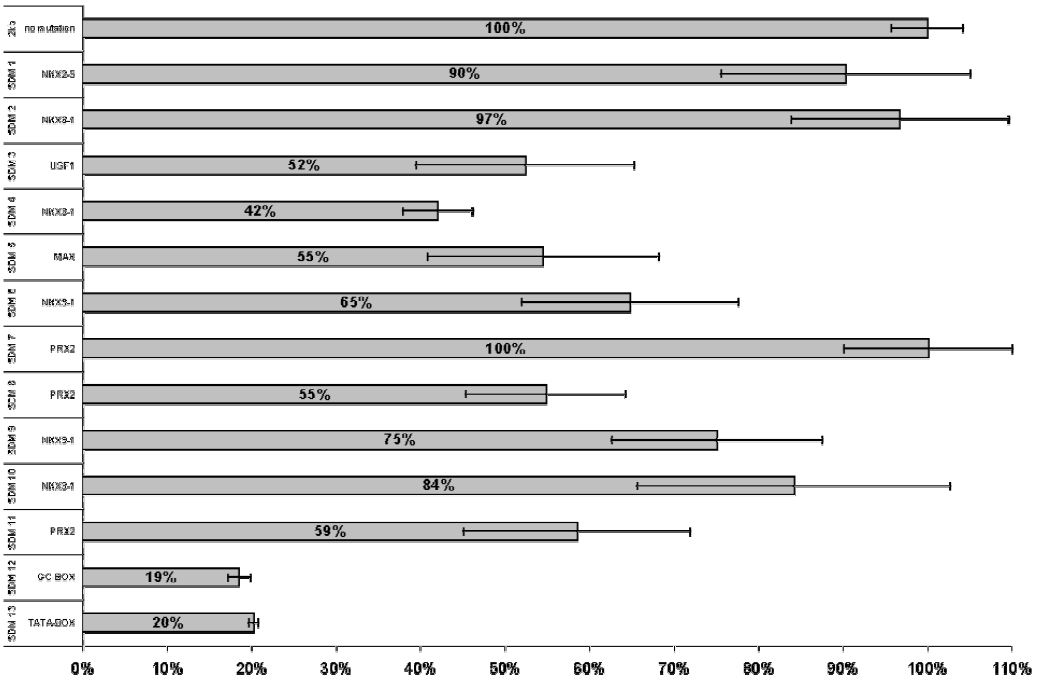

Fig. 42 Effect on activation activity of different cis-acting elements in the TRPM8 promoter. The 13 TRPM8 promoter (1.9 kb) constructs were designed each carrying a deletion of 6-9 base pairs specific for one ore more transcription factor binding sites shown in Fig. 41. The mutated constructs were located in front of a luciferase reporter gene in the pGL3-vector. LNCaP cells were transfected for 24 h with the wild-type promoter and the 13 constructs carrying the specific site directed deletions. Activation potential of each deletion construct is shown in relative expression to the wild type promoter (100%).

2.10.5 Androgens enhance transcription of TRPM8

In order to evaluate if TRPM8 is regulated by androgens, LNCaP cells were cultured in steroid reduced medium and subsequently treated with 1nM, 10nM and 100nM of the androgen R1881. After 24 hours of treatment mRNA was isolated and RT-PCR was performed using gene specific primers for TRPM8, KLK3, NKX3-1 and MYC. The results were adjusted to the house keeping gene SDHA (succinate dehydrogenase complex, subunit A) and shown as relative expression to solvent ethanol (Fig. 43). R1881 enhances the transcription of TRPM8 nearly 90 times in LNCaP cells whereas KLK3, the gene which codes for PSA and NKX3-1 are upregulated 30 times and 6 times, respectively. The expression of the transcription factor MYC is not regulated by androgens.

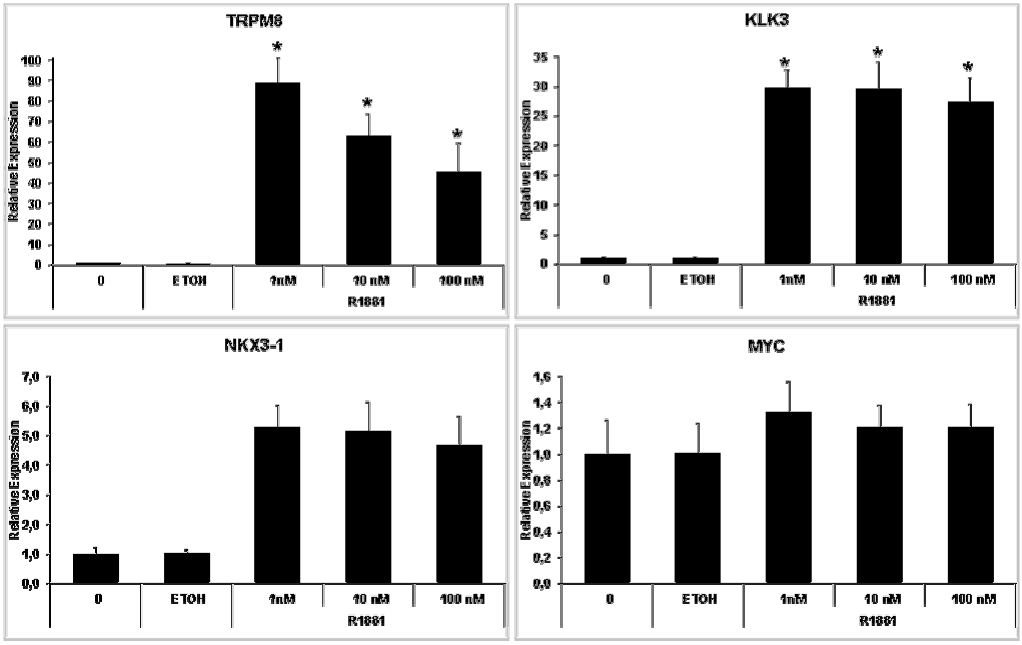

Fig. 43 Effects of androgen on the TRPM8 expression. KLK3, NKX3-1 and MYC expression in LNCaP cells. * indicate the significant upregulation in expression compared to ethanol, the solvent of R1881, with p values at least < 0.001 (t-test).

This was also an interesting finding, especially as the 1.9 kb fragment of the promoter did not show any androgen responsive element (ARE). Additionally, it should be analyzed if androgens alter the activation potential of the 1.9kb-pGL3 promoter construct. Therefore LNCaP cells were treated in the same manner as described above, but additionally when treatment of androgens started, cells were transfected with the 1.9-pGL3-promoter construct and the phRL-TK vector as a transfection efficiency control. After 24 h cells were lysed and luminescence measured. Results are shown in Fig. 44: The activation of the 1.9-pGL3 promoter compared to the pGL3-empthy vector was approximately 8 times, however the addition of R1881 did not activate the promoter significantly when results were adjusted to the empty pGL3 vector (data not separately shown). Thus androgens do not enhance this part of the promoter.

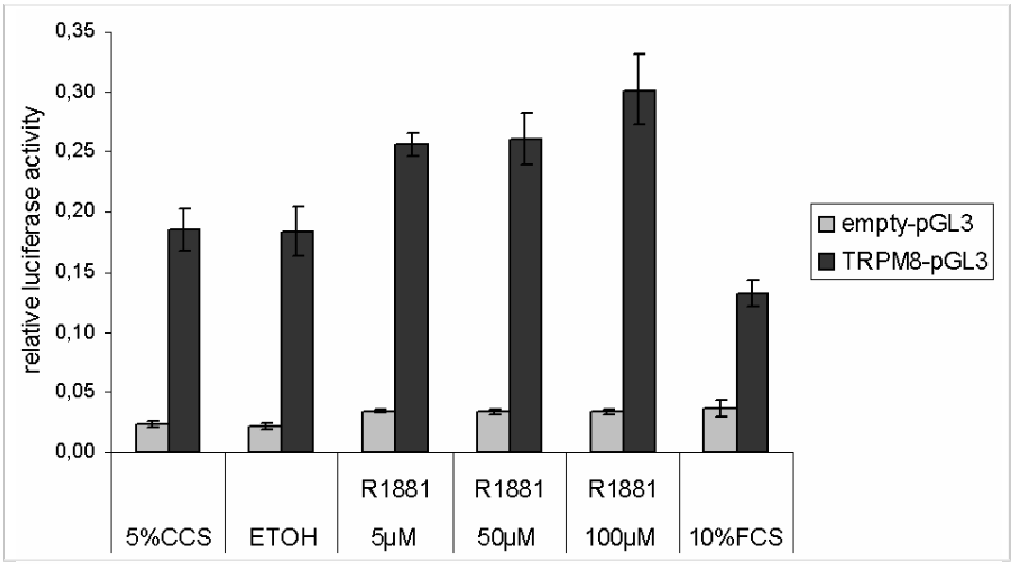

Fig. 44 Influence of androgens on the promoter activity of TRPM8. LNCaP were cultured in androgen depleted serum before the experiment. The 1.9kb promoter of TRPM8 was cloned in front of the luciferase reporter gene into the pGL3-basic vector (promega). Upon transfection with the TRPM8 promoter, cells were treated with R1881, 10% FCS or ETOH. Analysis was performed after 24h of treatment. Data is shown as relative luciferase activity to the transfection control plasmid phRLTK-null.

2.11 CORRELATION OF TRPM8 TO GRADING AND STAGING OF PROSTATE CANCER

Affymetrix gene chips experiments were analyzed for correlation of TRPM8 expression to the Gleason grading and the TNM staging system. Patients' PMQ values for TRPM8 and PSA were grouped by staging and grading and the median of each group was calculated. Results showed that TRPM8 mRNA expression increases with Gleason sum from normal tissue to a Gleason sum of 8, but mRNA levels fall to nearly normal levels in high grade tumors (Gleason sum of 9) (Fig. 45 A and B). Correlating TRPM8 expression to the TNM staging system showed similar results. Expression of TRPM8 first rise in early stage tumors compared to normal tissues, but when the tumor has extended through the prostatic capsule into the seminal vesicles (T3) TRPM8 levels fall. Stage 4 cancers, which are characterized by the spreading of the tumor into the bladder neck, show further reduced expression of TRPM8 compared to T3 tumors (Fig. 45 C and D). On the other hand, PSA levels do not show any correlation to either staging or grading.

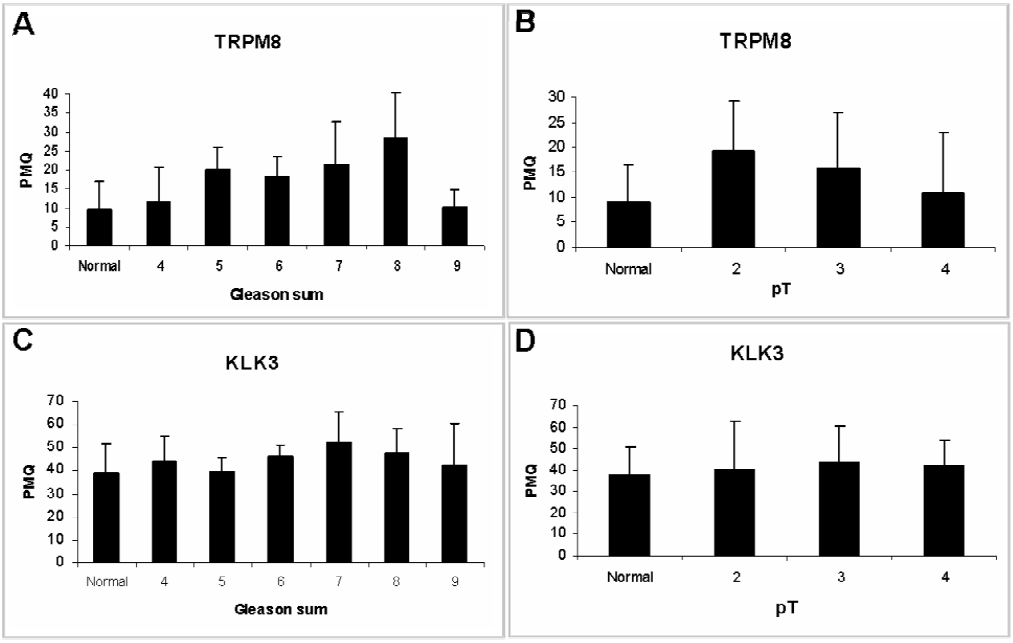

Fig. 45 Microarray results of TRPM8 and KLK3 in correlation to Gleason sum and TNM staging (pT). Data is shown as the median of PMQ for each patient group. At least 3 patients represent one group.

3 Discussion

3.1 MICROARRAY EXPERIMENTS AND PROSTATE CANCER PROFILING

Microarrays represent a very effective technique for the simultaneous screening of many genes. They are ideal tools for the identification of new molecular markers and therapeutic targets in cancer and other diseases. In this study a special custom designed array was used to screen for new genes differentially expressed in prostate cancer. It was possible to identify more than 100 genes over- or underexpressed. Comparing these results with published data sets revealed a general congruence: two thirds of the genes found in this study were also found by other prostate cancer profiling studies [Welsh, 01; Rhodes, 02; Pilarsky, 03; Magee, 01; Luo, 01; Dhanasekaran, 01]. Only a small fraction (less than 15%) was found in this study, but not in others, although the probesets for the genes were present on their chips. This is an interesting finding as one would expect more discrepancies between the studies based on so many different parameters such as patient material, treatment of the material (microdissected or bulk) and the used chip technique (spotted or synthesized). For example, in this study the very time consuming microdissection of the tissue was performed, which but not in the others. However, still similar results were obtained. This leads to the conclusion, that microdissection may not be as important for prostate cancer profiling as previously expected.

A meta-analysis of gene expression profiles in prostate cancer [Rhodes, 02] showed that the influence of the chip technique used was also negligible as two spotted studies [Dhanasekaran, 01; Luo, 01] showed approximately similar results in comparison with a synthesized study of Welsh *et al*. [Welsh, 01]. The main reason why each study found approximately 20% varying differentially expressed genes is the fact that in each study different chips were used, representing different genes. Indeed, TRPM8 could only be identified in this study because it was present on the metaGen Cancer-Chip. None of the other studies performed between 1997 and 2001 represented this gene on their chips. In the future gene profiling studies of cancers (or of other diseases) will not come up with many new genes as the human genome is more or less sequenced and approximately 95% of the genes known are present on whole human chips (HG-U133 Plus 2.0) representing 47,000 genes (Affymetrix, Santa Clara, CA). The next challenge will be the characterization of these genes in a more detailed way, looking closer at splice variants and in a next step at the protein level.

3.2 OVEREXPRESSION OF TRPM8 IN PROSTATE TUMORS

Chip experiments revealed that TRPM8 was overexpressed in 56% of all prostate tumor patients, ranking on position 4 of the most overexpressed genes. In order to approve the results from the microarray experiments Real Time PCR was performed. The analysis showed that TRPM8 was indeed highly overexpressed in prostate cancer patients (64%). The most striking and important feature concerning the potential of TRPM8 as a drugable target was seen in experiments characterizing its expression among diverse normal tissues. Northern and dot blotting as well as *in situ* hybridization experiments over a wide range of tissues and patients revealed an exclusive expression of TRPM8 in the prostate, with no expression detectable either in any other normal human tissue, or in any other cancer tissue, with notable exception of the very rare neuroendocrine tumors. These findings stand in contrast to results from Tsavaler *et al*. [Tsavaler, 01] who indicated that TRPM8 is expressed at least in trace amounts in normal tissues such as testis, lung, breast, thymus and lung. They also showed that TRPM8 is expressed in different other primary cancers, such as melanoma, colorectal carcinomas and breast carcinoma. The reason for this discrepancy may be due to the different probes used for hybridization. Tsavaler *et al.* used a 342 bp fragment of which they do not specify the exact localization in the TRPM8 gene. But as they isolated this clone from a cDNA library which was prepared from an mRNA pool it is most likely that the fragment is located near the poly-A tail. In first experiments performed in this study using a short probe derived from the 3' UTR of TRPM8 it was also observed that transcripts- although different in size- were seen in other organs such as liver and brain (Fig. 46 B). When hybridization was done with a 2.7 kb probe of the open reading frame of TRPM8 the exclusive expression in prostate tissue could be seen as shown in Fig. 46 A. This fact explains also the discrepancies in *in situ* hybridization experiments seen in this study compared to results from Tsavaler *et al*. However, it can not be excluded that the expression seen was due to some unspecific binding of the probe. On the other hand results from this study and literature search revealed that often many isoforms of a gene exist which exhibit different expression patterns. In a very recent and interesting finding it could be proven that the differential expression of a gene was only due to just one specific exon [Gandini, 03]. They showed that only exon 4 of the prostate cancer antigen 3 (DD3) is differentially expressed in prostate tumors, whereas exons 1-3 are uniformly expressed in both tumor and normal tissue. Interestingly, this gene was the most differentially expressed gene found in this study by microarray experiments (77% overexpressed; Tab. 1). These findings do not show only how carefully one has to choose the probe for hybridization experiments, but it also indicates that a more detailed experiment design will reveal much better insight in the understanding of the complexity of gene expression.

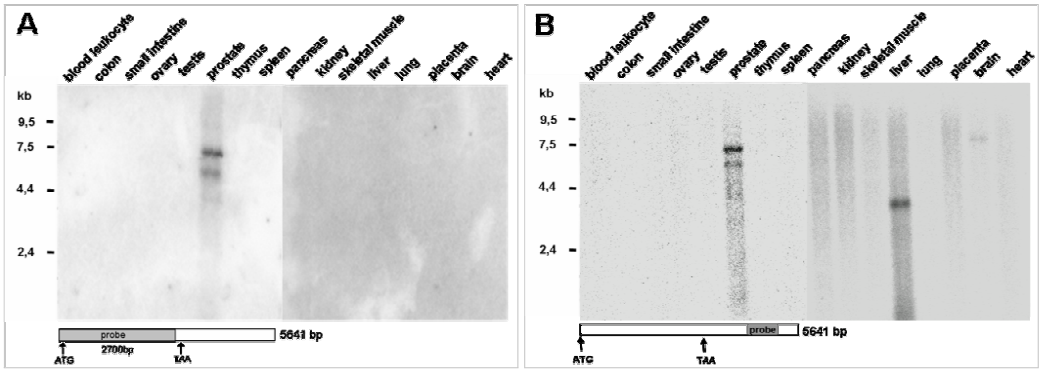

Fig. 46 TRPM8 Northern blot using 2 different probes for hybridization. A) Northern blot using a 2.7 probe from the 5'-end of the TRPM8 gene. **B)** Hybridization with a probe from the 3' end of TRPM8. All other conditions were exactly the same.

3.3 TRPM8- A FUNCTIONAL CALCIUM CHANNEL

TRPM8 belongs to the transient receptor potential family. It has a six membrane spanning domain with both COOH and NH_2 termini located intracellularly. In FRET experiments it was possible to demonstrate for the first time that TRPM8 subunits homomultimerize. Most likely they form tetramers as it was shown for several TRP channel such as TRPV1, TRPC4 and TRPV5 [Schaefer, 02; Strubing, 01]. Coexpression of C-terminal fusion proteins (TRPM8-CFP and TRPM8-YFP) yielded FRET efficiencies of nearly 16%. Comparing these data with published FRET efficiencies, the interaction of TRPM8 subunits is quite strong. For example FRET efficiencies of approximately 9% and 8% were detected for TRPC4α and TRPC4ß, respectively [Schaefer, 02]. The TRPV1 altered FRET efficiencies of 18.5%.

Two studies analyzing the TRPM8 orthologs in mouse [Peier, 02] and rat [McKemy, 02] showed that TRPM8 is activated by cold stimuli and cooling agents such as menthol and icilin. Following these findings it was possible to show that the human TRPM8 could be activated by a cooling agent, too. The human TRPM8 channel could be activated strongly with icilin, which was followed by a large Ca^{2+} invard current into the cell. The mechanism of TRPM8 activation by icilin is not yet known. Either it is directly activated through conformational changes or by activation through a second messenger pathway [Peier, 02]. The observations made by Peier *et al.* and also in this study reveal that TRPM8 is activated immediately after exposure to the agent suggesting a direct gating mechanism.

TRPM8 is the only member of the TRPM8 subfamily for which activation by cooling agents and by cold has been shown. Although all TRPM proteins are Ca^{2+} permeable channels, their mode of activation is different. For example TRPM2 is activated by H_2O_2 [Kraft, 04; Wehage, 02], TRPM1 by switching the cells from Ca^{2+}-free to Ca^{2+} containing medium [Xu, 01]. TRPM5 could be activated by the depletion of intracellular calcium stores [Perez, 02].

3.4 TRPM8 EXPRESSION IS REGULATED BY ANDROGENS

Analysis of TRPM8 expression among different cell lines revealed exclusive expression in the LNCaP cell line. Neither any of the 4 additional prostate cell lines nor any of the other cell lines derived form various human tissues express TRPM8. One of the profound features of the LNCaP is their androgen dependency. Thus it was analyzed whether TRPM8 was regulated by androgens or not. Indeed, Real Time PCR experiments performed on LNCaP cells incubated with different concentrations of the androgen R1881 revealed a 90 times upregulation of TRPM8. This is a new and interesting finding especially as KLK3, the gene coding for PSA, could just be activated by androgens up to 30 times. However, absolute KLK3 mRNA levels in untreated and in treated cells were higher than TRPM8 mRNA levels. This could be many reasons for these findings: 1. KLK3 is additionally co-activated by other factors, 2. the AR activation of transcription of the KLK3 gene is more pronounced, due to a higher specific transcription factor binding site to the KLK3 promoter, 3. Expression of TRPM8 is regulated by specific other transcription factors, or 4. the AR activates the TRPM8 gene to a lesser extent which might be due to less specific TF-binding sites in the TPM8 promoter. A study from this summer supports the finding that TRPM8 is androgen regulated [Henshall, 03]. They grew the androgen prostate cancer xenograft LuCaP-35 subcutaneously in nude male mice. Tumor bearing animals were castrated and tumors were harvested at several time points after castrations (0 -100 days). They showed that TRPM8 expression levels were high in mice on day 0 – 2 after castration but not 5 -100 days post castration. Further, TRPM8 mRNA expression correlated significantly with KLK3 expression in the same mice (Person P = 0.80).

Collectively, these data suggest that TRPM8 is regulated by androgens, a mechanism by which it could also be regulated in human prostate tumors.

3.5 TRPM8 PROMOTER

Taking advantage of the specific expression of TRPM8 in the prostate, it was analyzed whether the TRPM8 promoter could be used for gene therapy. The strategy was to clone the TRPM8 promoter in front of the sequence of a certain toxin, such as diphtheria toxin A into a viral vector used for gene therapy. Expression of the toxin would thus be restricted to the prostate as the transcription of the toxin would be under the control of the tissue specific TRPM8 promoter.

In order to analyze whether the promoter could be used following this approach the 1.9 kb fragment of the 5' flanking region of the transcription start site of TRPM8 was analyzed *in silico*. It was possible to identifying multiple transcription factor binding sites such as PRX2, NKX3-1, NKX2-5, USF1 and MYCMAX. The most striking feature is the high abundance of binding sites for homeobox genes such as PRX2, NKX3-1and NKX2-5.

In reporter assays cloning the TRPM8 promoter in front of a luciferase reporter gene it could be demonstrated that the 1.9 kb-promoter of TRPM8 was able to activate the transcription significantly, ranging from 3.9 times in HEK293 cells to nearly 14 times in DU145 cells. In LNCaP cells the activation level was about 9 times. This was an unexpected result as in other experiments it could be shown that expression of TRPM8 was restricted to the cell line LNCaP. Therefore it was expected that the promoter would be strongest activated in this cell line. Concluding, the identified promoter region does not represent the functional element for prostate specific expression, especially as androgens, in this case R1881 was not able to enhance the basal transcription activation. But as androgens definitely raised transcription of TRPM8 up to 90 fold in R1881 treated LNCaP cells, there must be enhancement through other mechanisms. Either, activation of TRPM8 is regulated by additional enhancer elements upstream or downstream from the transcription start site or the activation is regulated by other mechanism than on the transcriptional level. Most likely the first assumption is correct: It could be shown for the FLOH1, which is largely regulated by androgens, that the basal activation of the promoter was greatly enhanced by a DNA fragment found in intron 3 of the FLOH1 gene[Warren D.W., 03]. Unfortunately they neither describe the basic promoter nor the DNA-piece from intron 3 precisely, but it indicates that activation of androgen responsive genes can be regulated by elements more than several 1000 base pairs away from the transcription start site.

Additionally, *in silico* analysis of the 30 kb upstream and downstream of the transcription start site of the TRPM8 gene showed many potentially androgen responsive elements (AREs). Although it was not possible to find any classical consensus sequences for AREs, several sequence altering the core base pairs for potential AREs could be identified. Additionally, some of the AREs were present in conserved regions which stress their importance. Further promoter studies including these binding sites will give further insights into the transcriptional regulation of TRPM8.

It was further possible to identify a highly conserved 172-bp element within the TRPM8 promoter, which functions as a transcriptional repressor. This is consistent with previous reports demonstrating that a NKX3-1 element can repress the activity of a basal promoter containing a multimerized NKX3-1 binding site when it was expressed in TSU-Pr prostate cells [Steadman, 00]. However, this observation was only made when artificial NKX3-1 was co-transfected: When the multimerized NKX3-1-reporter construct was transfected alone, the promoter activity was even enhanced. In this study the repression was independently of exogenous TF indicating that this element is a strong repressor; especially as repression could also be seen in non-prostate cells such as the HEK293 kidney cells. Experiments co-expressing the 172-pGL3 construct with NKX3-1 will show if this could further reduce the activation potential. Another possibility to analyze the function of this repressor would be to exclude the 172-base pair fragment from the 1.9 kb construct. It is likely that this would enhance the activation potential of the promoter.

3.6 EXPRESSION OF TRPM8 IN NEUROENDOCRINE TUMORS

Microarray analysis of lung cancer patients revealed that one lung carcinoma sample expressed TRPM8. The analysis of the clinical data of all 172 lung cancer samples indicated that this patient was the only one with a 10% neuroendocrine infiltration. As this finding could be pure coincidence, Real-Time PCR was performed including a 100% neuroendcrine tumor (not used in chip experiments) of the lung and as a negative control an adenocarcinoma of the lung. The result was convincing: TRPM8 was more than 60 and 900 times overexpressed in the 100% and 10% neuroendocrine tumors, respectively. Most likely that the normal tissues did not have any expression at all and the trace expression seen was just due to contamination. RT-PCR experiments with cell lines of neuroendocrine origin supported these results: TRPM8 was expressed also in LCC18 (colon), QGP1 (pancreas) and BON1 (pancreas) cells.

3.6.1 Expression of TRPM8 in neuroendocrine and prostate cells

The finding that TRPM8 is expressed in neuroendocrine cells (NE) and prostate tissues raises the question, whether both cell types have something in common. For example, is it possible that neuroendocrine (NE) cells are present in the prostate? The answer is yes. NE cells represent, beside the basal and the secretory cells, the third form of epithelial cells of normal prostate epithelium [Abrahamsson, 96]. They are located in all regions in the prostate and are present in normal, hyperplastic and dysplastic prostate tissue. NE cells have a complex appearance with irregular dendrite like processes extending between adjacent epithelial cells. Abrahamsson *et al*. summarizes the cytological and histological patterns of NE cells as follows:" Ideally, a NE cell is defined as a cell of neuronal or epithelial type that fulfills all or most of the following criteria: it contains secretion granules; its secretion is essentially derected towards the blood, …, and is immunoreactive to antisera against neurone-specific enolase or chromogranin A or other NE markers".

This "nerve like" appearance could also be observed when LNCaP cells were cultured for a period longer than 10 days in steroid deprived medium (own observation). Therefore it might be possible that TRPM8 is expressed in the prostate from neuroendocrine cells. But, are neuroendocrine cells amplified in prostate tumors, which may solve the question why TRPM8 is overexpressed in neoplastic tissue? The answer is again yes. It was shown that neuroendocrine differentiation in prostatic adenocarcinomas is associated with a poor prognosis [Bostwick, 02]. The most common form observed in prostate carcinomas is a focal neuroendocrine differentiation, which may be pronounced in approximately 10% of adenocarcinomas. Further, it could be shown that NE are positive for a nuclear located (and thus fuctional) androgen receptor [Nakada, 93]. In another study, Singh *et al*. showed that Cromogranin A, which is a marker for NE cells, is significantly overexpressed in prostate tumors. These findings support the speculation that TRPM8 might be expressed in neuroendocrine cells of the

prostate, but double staining of TRPM8 and neuroendocrine markers such as Chromogranin A or serotonin will have to be performed in the future.

3.6.2 Expression of TRPM8 in neuroendocrine cells and cells from the nervous system

The mouse and the rat orthologes of TRPM8 were isolated from RNA of DRG (dorsal root ganglia) and from trigeminal neurons of newborn rats, respectively [Peier, 02; McKemy, 02]. In humans TRPM8 it is expressed in the prostate with elevated levels in early stages of prostate cancers. All three orthologes (mouse, rat and human) can by activated by cooling agents such as icilin, menthol or by temperatures below 28°C. Looking at these characteristics, is there a regulation link between expression in prostate cancers and neuronal cells? Most likely, cold or cooling agents are not the biological stimulus of TRPM8 in the prostate. But are androgens expressed in neuronal cells? Most likely they are not. One possibility is that the transcriptional regulation of TRPM8 is completely different in these three species. For example it could be shown in this study that the mRNA of the mouse orthologe misses the first exon of human TRPM8, but alters 4 additional exons at the 5' end, which so far were not seen in humans. Thus the promoter regulating the TRPM8 gene transcription in human and mouse might be completely different leading to different activation of the transcription.

Another point to be discussed is the question why TRPM8 expression is regulated by androgens, but channel activation occurs through cooling agent such as icilin? This is another point why the TRP family has been denoted as "a very interesting and versatile family" [Montell, 02] .

3.7 TRPM8 EXPRESSION CORRELATES WITH DISEASE PROGRESSION

In situ experiments on prostate cancer patients in this study revealed that TRPM8 is expressed moderately in all normal prostate cells, strongly enhanced in PIN and in adenocarcinomas. However it was not possible to correlate *in situ* expression with disease progression; neither the Gleason Grading System nor the TNM System showed any significant correlation to TRPM8 expression. Locally TRPM8 is predominantly expressed in basal epithelial cells, which is conform to results from Tsavaler *et al*. [Tsavaler, 01].

Affymetrix gene chips experiments were analyzed for correlation of TRPM8 expression to Gleason grading or TNM staging. Results showed that TRPM8 mRNA expression increases with Gleason sum linearly from normal tissue up to a Gleason sum of 8, but mRNA levels dropped significantly to nearly normal levels in highly undifferentiated tumors (Gleason sum of 9). Correlating expression of TRPM8 to the TNM staging system showed similar results: Expression of TRPM8 first rise in tumors of early stages (N - T2), but when the tumor has extended through the prostate capsule into seminal vesicles (T3), TRPM8 levels fall. In cancers stage 4, when the tumor has spread further into the bladder neck or other nearby tissues, expression drops even more. Collectively,

TRPM8 increases at the beginning of the disease, but in very undifferentiated tumors which have extended through the prostate capsule expression decreases significantly. *In situ* hybridization further supported these findings as it was observed in cases where high grade tumors and low grade tumors were present in the same specimen, that TRPM8 was lost in undifferentiated tumor cells (GGs 9-10).

The next question to be answered was whether TRPM8 also correlated with PSA-relapse after radical prostatectomy. Interestingly this was not the case. In July this year a study was published showing that TRPM8 is a good prognostic marker of PSA-relapse [Henshall, 03]. They analyzed (using also Affymetrix GeneChip) 17 patients with a PSA relapse after radical prostatectomy, compared to 55 patients who remained free of PSA relapse after surgery. It indicates that patients with low TRPM8 expression prior to radical prostatectomy have a higher risk of getting a relapse of PSA. According to this study until now 8 patients out of 52 had a relapse of PSA, but it was not possible to correlate this to TRPM8 expression. It may be assumed that the number of patients was too small and/or the follow-up time too short in order to find any correlation, but the other study was not much bigger for statistical significance. Thus it remains to prove in larger studies whether TRPM8 is a prognostic marker for PSA relapse.

3.8 TRPM8- TARGET FOR TREATMENT OF PROSTATE CANCER

Prostate specific antigen (PSA) and the digital rectal examination (DRE) are the two major screening parameters for prostate cancer. But neither PSA nor any of the other prostate cancer specific markers available today meet the requirements of a good marker: sensitive, specific, providing prognostic information, and indicating post treatment progression or cure. For example prostatic acid phosphatase (PAP) has been used extensively for diagnosis, staging and monitoring of prostatic cancer in the last century, but it is ineffective for screening of prostate cancer as it has a low positive predictive value, a low specificity and sensitivity. Also PSA, it is specific for the prostate, but not for prostate cancer as it is expressed in all stages of cancer [Caplan, 02]. It tends to increase with age and rises in men with evidence of benign prostatic hypertrophy. Additionally, preoperative PSA cannot be used to predict capsular penetration or seminal vesicle invasion. Neither PSA nor PAP are able to predict progression in adenocarcinomas of the prostate following radical prostatectomy [Sauvageot, 98]. But is TRPM8 at better diagnostic marker than PSA? Most likely it is not. 1. TRPM8 mRNA expression rises and falls with progression of the disease, which makes it difficult for the pathologist to distinguish (at least alone) whether the patient alters a tumor of very high or very low grading or staging. 2. The findings in this study were based on RNA data, thus pathological classification would be very time consuming and expensive. Whether TRPM8 expression could be useful for immunhistochemical classification remains to be demonstrated. 3. As TRPM8 is not a secreted protein, it will not be measurable in the blood, excluding it for the usage as a general screening parameter.

On the other hand TRPM8 is an extremely good target for the development of a drug. It was shown in this study that a drug against TRPM8 would only affect prostate tissues and neuroendocrine tumors, as TRPM8 is exclusively expressed in these tissues. The data from this work at least proves that TRPM8 mRNA could be a good target for an antisense drug. Although it was not part of the study to analyze the expression of TRPM8 on the protein level it could be proven in experiments done in parallel within the company that TRMP8 is also highly overexpressed on the protein level in prostate cancer patients. Further, mRNA expression correlated with protein expression in prostate cancer patients. Thus therapeutic approaches using antibody and small-molecule are also possible. But would it be enough to develop a therapeutic that inhibits the function of TRPM8 and thus prevents the cancer from growing or even better diminishes it? Is TRPM8 a promotor of tumor growth? Most likely it is not. In experiments overexpressing TRPM8 in cell lines, the division of the cells was neither enhanced nor did the cells have a different morphology. Thus the development of a therapeutic must focus on the design of a drug which binds to and destroys the tumor cells. That could be achieved either by using modified antibodies delivering toxic or modulatory payloads (small-molecules, radionuclides and enzymes) to the cancer cell or by the specific delivery of a radioactive payload carried on a small-molecule[Chang, 02]. That this approach is feasible has been shown over the years since it was introduced more than 20 years ago

3.9 SPLICE VARIANTS OF TRPM8

In this study more than 10 splice variants (SV) of TRPM8 could be identified using an *in silico* approach and RT-PCR experiments. All of them were as differentially expressed as TRPM8 itself, some of them exhibited even a significantly higher differentially expression in prostate tumors. Especially the regulatory RNA, located on the opposite strand of TRPM8, exhibited an 80% overexpression of TRPM8 in prostate tumors as analyzed by RT-PCR experiments. The identification of splice forms of a member of the transient receptor potential family is not unique. It is the fourth TRPM family member for which isoforms have been identified. Up to now splice variants of TRPM1 (MLSN) [Xu, 01], TRPM2 [Zhang, 03] and TRPM5 (MTR1) [Prawitt, 00] have been described. In this study splice variant 16b was more closely examined. It has a truncated C-terminus leading to truncated transmembrane domains with loss of the functional pore. Interestingly, in addition to 16b of TRPM8, isoforms of TRPM1 and TRPM2 also have a deletion of the C-terminus. The three short forms alter different numbers of transmembrane domains. TRPM1 is devoid of all, 16b has one and TRPM2 has two transmembrane segments. The short form of TRPM1 is uniformly distributed in the cytoplasm, whereas the long form localizes in the plasma membrane. Published data show that the short form of TRPM1 suppresses the function of the long form by inhibiting the transportation of the long form to the plasma membrane [Xu, 01]. By contrast the TRPM2 short form does not alter the localization of the long form. It inhibits the function of the long form by an unknown mechanism [Zhang, 03]. The data in this study

show similar results as shown for TRPM2. 16b does not alter the location of TRPM8 (at least not visible in confocal microscopy experiments), but shows -at least partially- an inhibiting effect on the activation of TRPM8 by icilin.

All of the study discribed above were performed *in vitro* overexpressing both, the long form and the short form through expression-vectors. No study is available which discribes functions of endogenously expressed splice forms of TRP channels. There will be further studies necessary in order to prove that the truncated transcripts are a) endogenously translated into proteins and b) expressed by the cell in a sufficient amount to alter the functions of the longer form. One reason why no publication is available might be the difficulty in finding working antibodies which selectively recognize only the truncated forms. In most cases the splice variants do not differ in a large number of amino acids. Thus the availability of a good epitope is very limited.

What might be the function of all the other identified splice variants? Do they have any functional properties? It is quite difficult to believe that all of the identified transcripts have a distinct function. But if so, does the abundance of spliced transcripts contribute to the malignant transformation? Most likely only a few of them have functional properties. The great majority might be overexpressed just because of diverse disregualtions in cancers.

Interestingly, most of the identified splice variants in this study were isolated from mRNAs derived from tumor tissues. This might indicate that not only the overexpression, but also the diversity of splice variants in tumors is increased. Real-Time PCR experiments as well as dot blot experiments revealed that some of these isoforms are much stronger differentially expressed as TRPM8 itself. That makes them excellent markers for the detection of prostate cancer.

Alternative splicing has been shown to be tightly regulated in a tissue and developmental- specific manner [Nissim-Rafinia, 02]. Therefore, changes in the absolute or relative expression of isoforms are expected to effect cellular functions which might be a contributing factor or cause to the development, progression or maintenance of cancer. Indeed, for many genes dramatic changes in alternative splicing patterns are associated with neoplasia and metastasis [Nissim-Rafinia, 02; Philips, 00]. In example WT1 (Wilms tumor), CD44 (renal, lung, gastric and urothelial cancers), BCL2 (prostate, lymphoma and gastric cancers) and FGFR2 (prostate cancers) are alternatively spliced genes found in cancers. BCL2L1 (formerly Bcl-x) a member of the BCL2 family, produces two alternative splice forms, one having a pro-apoptotic and one having an anti-apoptotic effect [Boise, 93]. It remains to find out what the reason for the upregulation of TRPM8 splice forms in prostate cancers could be. Four explanations are possible: First, mutation in the basal cis-splicings sides might be mutated. Second, mutation in auxiliary ISE/ISS or ESE/ESS elements might lead to the inappropriate expression of the isoforms. Third and fourth, *trans*-acting factors of the basal and the auxiliary splicing machinery are defective.

Most likely the overexpression of TRPM8 splice variants is due to changes in *trans*-acting factors of the auxiliary splicing machinery as mutations in *cis*-elements of the splicing machinery can not explain the immense diversity of

splice variants as demonstrated especially in splice variant 16b. Point mutations in the basal or auxiliary cis-elements would only lead to a few alterations in splicing, thus only a handful of new isoforms would be expected to be seen. Mutations in proteins functioning in the basal *trans*-system of the splicing machinery would cause all genes to be alternatively spliced, which is not very likely as this mutation would be lethal for the organism. Support for the theory of the alterations in the auxiliary trans-splicing machinery is coming from literature. It has been shown that tumor cells express high levels of a broad spectrum of a group of splicing factors which are members of a conserved family of proteins. These factors bind to the active sites of RNA polymerase II transcription and thus function as key regulators of alternative RNA splicing, whereas preneoplasias often express only a sub-set of the family [Stickeler, 99]. The major group of these proteins belongs to Serine/arginine-rich (SR) proteins [Zahler, 92; Fu, 95; Graveley, 00]. They have dual functions and serve as splicing enhancer or splicing repressor proteins, depending on where they bind in the pre-mRNA reviewed in [Akusjarvi, 03].

Although the majority of the isoforms of TRPM8 expressed in prostate tumors are most likely due to splicing defects resulting in unnatural splicing with no biological relevant functions, some of those splice variants might be of biological significance. In Northern blot experiments two isoforms of TRPM8 could be identified: one longer transcript as TRPM8 with a length of 7.3 kb and a shorter one with a length of 4.1kb, but neither of these matches the length of the transcripts identified in prostate tumors.

In conclusion it can be said that the splice variants found in this study are extremely good markers for the detection of prostate cancer. They would be good targets for a pay load based therapeutic approach. Their potential for being a target for a small-molecule has to be analyzed in further studies, as their contribution to malignant transformations has not yet been proven.

4 Outlook

In the last years the development of target drugs for the treatment of cancers has dramatically increased, a progress that is likely to continue in the future. It is obvious that targeting molecular abnormalities has considerable advantage over unspecific systemic drugs such as the chemotherapy. They are more specific, thus less toxic, and more effective in the treatment of cancer [Stockwin, 03]. It seems possible that targeted drugs will be used in association with existing medical, surgical, and radiotherapeutic therapies and will play an important role in the aim of curing cancer.

In the present work it was possible to identify TRPM8, as a perfect target for a specific prostate cancer therapy.

The focus in future experiments will be the development of specific drugs targeting TRPM8. It is hoped and expected that these therapeutics will lead to new and promising treatment forms of prostate cancer.

5 MATERIALS AND METHODS

5.1 METG001A CHIPDESIGN

A custom designed oligonucleotide microarray based on Affymetrix technology (Affymetrix, Santa Clara, CA) was developed by metaGen Pharmaceuticals GmbH in order to profile different cancer entities. This chip contains about 6200 probe sets which represent roughly 3.000 genes. Nearly half of the sequences were genes to be shown as overexpressed in various tumor entities derived from EST mining approaches [Schmitt, 99]. The other sequences are known tumor associated genes, sequence-tagged site (STS) markers and sequences derived from protein motives.

The identification of genes overexpressed in tumors for the chip design was based on the counting of expressed sequence tags (EST) in different tissues and diseases. The sequences were derived from public[9] and proprietary (Incyte Genomics, Palo Alto, CA, USA) databases. About 4 million ESTs were presented in both databases at the time of the study, which were sorted for tissues specificity and into pairs of benign and cancer tissues.

5.1.1 Automated extension of cDNA sequences (AUTEX)

ESTs are single pass-reads from randomly selected cDNA clones. They are approximatly 500bp in length. Of the 4 million ESTs screened, many represent the same gene covering different sections of the gene. In order to represent each gene only by one probeset on the chip these ESTs were assembled using the in house AUTEX (automated extension of cDNA sequences) algorithm [Schmitt, 99]. First, a BLAST (Basic Local Alignment Sear Tool) program was used to search for ESTs which represented the same gene using dbEST and Incyte databases. Second, alignment of these ESTs was performed to elongate the sequences. These contigs were again blasted to the EST databases. After repeated rounds it was thus possible to receive the maximal length of a contig representing the sequence of a gene.

5.1.2 Electronic Northern

The assembled cDNA contig from the AUTEX program was then analyzed for its distribution among these 4 millions ESTs. The number of ESTs matching to each sequence originating from one EST pool was counted and normalized to the EST pool size for each tissue. Fisher's exact test was used to assess the significance of differential expression among tissues and between normal and

[9] http://www.ncbi.nlm.nih.gov/dbEST/

cancer tissues. A gene was defined as differentially expressed at a p- value of < 0.05. Fig. 47 summarizes this *in silico* approach.

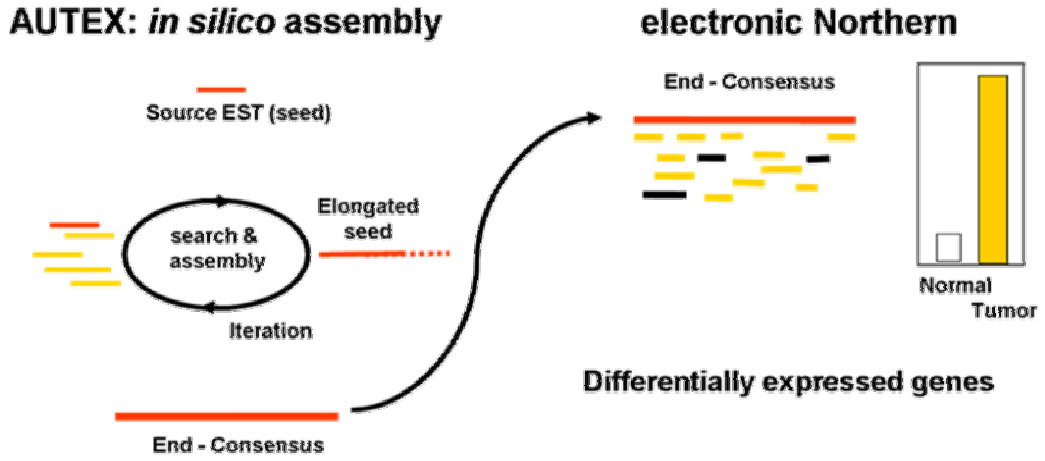

Fig. 47 Schematic drawing of the gene assembly program and the in silico expression profiling in cancers taken from [Schmitt, 99].

5.2 GENE CHIP ANALYSIS

5.2.1 Tissue Collection

Prostate cancer and normal tissue specimens were obtained from 52 patients undergoing radical prostatectomy for clinically localized prostate carcinoma at the Department of Urology at the University Hospital Charité from 1998 to 2001. Patients aged 47 - 73 had a Gleason score 4 -9 and a tumor stage T2a - T4. Preoperative PSA levels were between 2-30 ng/ml. The prostatectomy specimens were sectioned by a pathologist immediately after surgical removal. Slices of tissue were flash frozen in liquid nitrogen and stored at -80°C.

5.2.2 Microdissection

Thirty serial frozen whole-mount sections of prostate tissue (10 µm) were air dried, briefly stained with hematoxylin and refrozen on dry ice. Every 10th slide (5 µm) was stained with hematoxylin, and eosin for documentation, and areas for microdissection were marked. The tumor was separated from stoma cells by microdissection with a needle (22G) under an inverted microscope (40 x magnifications). Tissues were collected in GTC buffer containing 2% β-Mercaptoethanol for further RNA preparation. All samples contained at least 90% tumor cells.

5.2.3 RNA preparation and amplification

Poly-A^+-RNA was isolated by magnetic separation using the Poly-A-tract 1000 kit (Promega, Heidelberg, Germany) according to the manufacturer's protocol. cDNA synthesis and repetitive linear amplification (3 rounds) was performed with minor modifications as described [Luo, 99]. Briefly, cDNA was synthesized by priming the RNA with the Affymetrix T7-oligo-dT promoter-primer combination (5`-GGCCAGTGAATTGTATACGACTCACTATAGGGAGGCGG$T_{24}$-3` at 100 mM) and the reaction was incubated at 37°C for 1h. From the resulting cDNA the second strand was synthesized by first digesting the RNA partially. The RNA remnants thus functioned subsequently as primers for second strand synthesis. In vitro transcription was performed using the Megascript kit from Ambion (Huntington, UK). From the generated aRNA a new first strand synthesis was initiated using 0.025 mM random hexamer as primer. The second strand was synthesized using the Affymetrix T7-oligo-dT promotor-primer at a concentration of 0.1 mM. Again in vitro transcription was performed. The cDNA of each round of amplification was tested by Real Time PCR for its integrity. cDNAs of low quality were excluded from further analysis. A third round of amplification was performed as described above with the difference that biotin labeled nucleotides (Bio-11CTP and Bio-16-UTP, ENZO, NY, USA) were incorporated into the RNA within the *in vitro* transcription reaction. After RNA fragmentation to 50–200 nucleotides, 15 µg of biotinolated RNAs were hybridized to the metg001A Cancer-Chip. The arrays were then processed on the Affymetrix fluidics station and hybridization signals were visualized using phycoerithrin-conjugated streptavidin (Molecular Probes, Eugene, OR). GeneChips were scanned at 570 nm using an Agilent GeneArray Scanner.

5.2.4 Data processing

Data processing was performed by the bioinformatics group within metaGen. Raw intensity values were extracted from .cel- files. For background correction the chip was partitioned into 16 tiles. Each tile the mean of the 2% probes with the lowest intensities was determined and subtracted from each probe value respectively. The background corrected probe intensity values were normalized by dividing them by the median value of all probes. A representative expression value for each probe set (PMQ-value) was generated by using the 75th percentile of the PM-intensities. For each probe set a p-value for a 'present call' was calculated by comparing the intensities of the PM and MM probes using the Wilcoxon rank sum test for paired data [Wilcoxon, 45]. To minimize technically caused data perturbation a model fitting algorithm was applied to the PMQ data. For this purpose an ideal expression profile was constructed by determining the median PMQ- value of all analyzed chips, thus representing a theoretical reference chip.

Expression data of each individual chip was compared to the reference chip by applying the data to a *xy* scatter plot, in which *x* is represented by the reference chip data and *y* by the individual chip data. A linear regression using 'Robust Statistics' rules [Hudson, 81] was performed, resulting in a linear equation

described by their parameters slope *m* and intercept *n*. Using these parameters the data was linear transformed, so that in the *xy* plot a slope m=1 and an intercept n=0 was reached. Since after application of this fitting approach non linear effects ('banana shape' distribution) were still visible a segmented algorithm was created. For that purpose the probe sets were separated into two groups by means of their predominant call (absence or presence, *p-value* >, < 0.05). Both groups were then fitted individually as described above. Finally resulting PMQ-values were normalized again by dividing them by the chip median PMQ.

5.2.5 Prostate Cancer Gene Expression Analysis

From the 6117 probe sets present on the meta001A chip 3023 were used to identify differentially expressed genes. The reasons why 50% of the probe sets were sorted out are multiple: first, 688 were sorted out because they represented positive and negative controls; second, some sequences represented not only one gene, but represented whole groups of genes; third, most of the probe sets had to be excluded because they represented the same gene, i.e. not one probe set represented one gene, but 2-4 probe sets. This was due to the chip design. The *in silico* search for differentially expressed genes compared expressed sequence tags (EST) from tumor libraries with ESTs from normal libraries. The information gained from these ESTs was usually restricted to approximately 500 base pairs of sequence information which usually did not cover one whole gene. As many ESTs overlapped it was possible to use the AUTEX program to elongate the sequence information of each gene. But for many genes it was not possible to find enough ESTs to cover the gene from the 5' end to the 3' end resulting in "EST gaps". Not knowing that these ESTs belong to the same gene one probe set per EST-consensus sequence had to be designed and added to the chip, which led in some cases to more than one probe set per gene. As more and more information from the human genome project became available these probe sets could be identified as belonging to the same gene. Another reason for double or triple represented genes is that some ESTs could not be assigned in the right orientation (5'-3') and therefore had to be added to the chip in both directions. From the remaining 3023 probe sets each represented a different gene which was proved by using the Unigene annotation tool blastN [10].

For prostate data analysis the quotient of the normalized PMQ value of each patient and probe sets (tumor/normal) was calculated when the gene was expressed in both tumor and normal samples (p- value < 0.05). For genes present only in either normal or tumor tissue (p- value >0.05) no fold change was calculated but marked as differentially expressed.

5.2.6 probeset comparison of metg001A and U113B

Normal and tumor tissues of prostate, pancreas, mammary gland, ovary and bladder were analyzed exclusively on the metg001A chip whereas samples of

[10] http://www.ncbi.nlm.nih.gov/UniGene/

tumor and normal tissues form lung and colon were at least partially run on the Affymetrix U133 GeneChips (HG-U133 Set). This Human Genome Set, consisting of two GeneChip arrays (U133A and U133B) representing almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes[11]. In order to ensure that expression data gained from both chips were comparable, probesets for TRPM8 on the metg001A (FFF991_at) and U133B (243483_at) were examined more closely (Fig. 48, Tab.5)

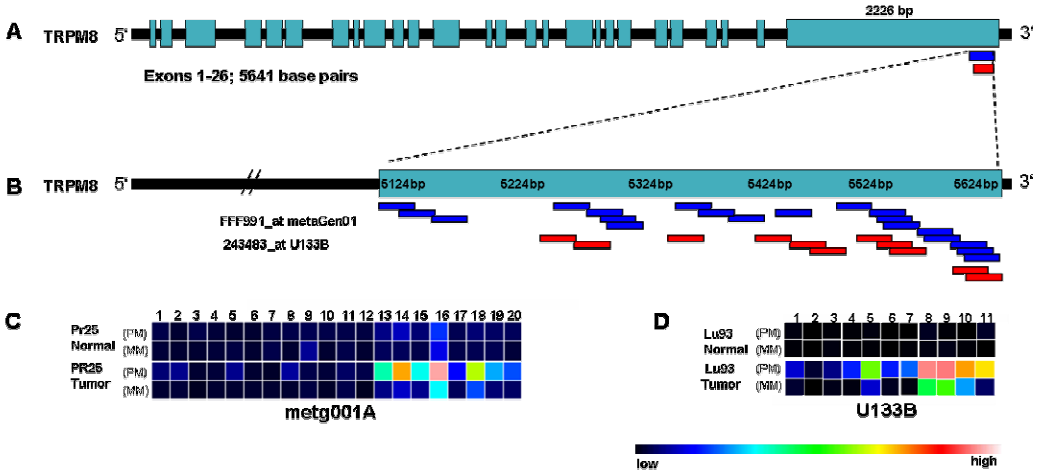

Fig. 48 Probeset distribution of TRPM8 on metg001A and U133B.

In general the metg001A chip shows 20 probesets per gene whereas the U133B chip represent a gene by a set of 11 probesets. The reduction of the number of probesets (the 25base pair/oligo remained the same) was due to results gained from first chip experiments showing that 11 probesets are sufficicent to represent a gene.

In both chips the probesets are located at 3' end of the TRPM8 gene. Most of the probesets from U133B and metg001A are overlapping as shown in red letters in Tab.5. Thus it can be concluded that the expression values gained from both chips are comparable. Looking more closely at the oligoprobesets of the metg001A chip it revealed that the probesets 6-10 alter 1-2 false bases, which makes them unusable for gene chip evaluation. But as the PMQ was taken from the 75th percentile the false probesets did not influence the results gained from the gene chip analysis.

Tab.5 Comparison of probesets for TRPM8 on the metg001A chip and the U133B chip. Blue bases indicate incorrect base pairs, red bases show the overlapping sequences in both chips. The grey shaded part points out the false probesets.

[11] www.Affymetrix.com

metg001A				U133B		
Probset number	Position TRPM8 (bp)	sequences of probeset FFF991_at	incorrect base	Probset number	position TRPM8 (bp)	Sequence of probset 243483_at
1	5124	TTAGTTTCTCAGCTTTGAATACACT		1	5241	TTTCCTTTTGTCTGGGCAGTAGTGA
2	5130	TCTCAGCTTTGAATACACTATAAAC		2	5264	GAAAATAACTACTCACAACATTCAC
3	5148	TATAAACTCAGTGGCTGAAGGAGGA		3	5343	GACAGAAAACTTGCCCAATACTGAG
4	5250	TGTCTGGGCAGTAGTGAAAATAACT		4	5397	AAATGTTTTCAACCCAGTTCATCTG
5	5274	TACTCACAACATTCACTATGTTTGC		5	5421	GGTGGATGTTTTTGCAGGTTACTCT
6	5280	CAACATTCACTATGTTTGCGAGGGA	A,A	6	5438	GTTACTCTGAGAATTTTGCTTATGA
7	5286	TCACTATGTTTGCGAGGGATTAACA	A,A	7	5501	GAACATACTTCTAATCAAAGGTGCT
8	5346	CAGAGAACTTGCCCAATACTGAGAA	A	8	5520	GGTGCTATGTCCTTGTGTATGGTAC
9	5358	CCAATACTGAGAAGCAACTTCCACT	G,T	9	5536	GTATGGTACTAAATGTGTCCTGTGT
10	5364	CTGAGAAGCAACTTCCACTAGAGAG	G,T	10	5568	GCACAACTGAGAATCCTGCAGCTTG
11	5406	TCAACCCAGTTCATCTGGTGGATGT		11	5579	AATCCTGCAGCTTGGTTTAATGAGT
12	5496	TGTATTGAACATACTTCTAATCAAA				
13	5514	AATCAAAGGTGCTATGTCCTTGTGT				
14	5520	AGGTGCTATGTCCTTGTGTATGGTA				
15	5526	TATGTCCTTGTGTATGGTACTAAAT				
16	5550	TGTGTCCTGTGTACTTTTGCACAAC				
17	5556	CTGTGTACTTTTGCACAACTGAGAA				
18	5568	GCACAACTGAGAATCCTGCAGCTTG				
19	5574	CTGAGAATCCTGCAGCTTGGTTTAA				
20	5580	ATCCTGCAGCTTGGTTTAATGAGTG				

5.3 REAL-TIME PCR

a) Expression of splice variants of TRPM8

First strand synthesis was done either from two times amplified aRNA or from freshly prepared mRNA. The cDNA was synthesized as described in the Affymetrix sample preparation. The cDNA generated from 1 ng RNA was used for the Taqman assay (Applied Biosystems, Weiterstadt, Germany). For quantitative PCR 1ng of cDNA was used in each reaction. The reaction was carried out in a 25 µl reaction volume containing 2.5 µl 10x SYBRGreen PCR Buffer (Applied Biosystems), 25 mM Mgcl2, 12.5 mM dNTP's (with dUTP) and 0.625 U Ampli Taq Gold (Applied Biosystems). The reaction was carried out on the "GeneAmp 5700 Sequence Detection System" (Applied Biosystems, Weiterstadt) according to the manufacturer's instructions. The primer sequences were designed with Primer Express software (Applied Biosystems) using the special Design program for TaqMan specific primers. Thus all PCR reactions could be carried out at the same conditions: Samples were denatured for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The analysis for each sample was done using the ΔΔct-Method according to the manufacturer (Applied Biosystems).

b) Expression of TRPM8, NKX3-1, KLK3 and MYC in R1881 treated LNCaP cells.

RT-PCR reaction was carried out using the QuantiTect SYBR Green (Qiagen, Hilden, Germany) following the manufacture's instructions. Briefly, RT and PCR reactions were carried out in the same reaction tube at the following conditions: 50°C for 30 min (RT), 95°C for 15 min, followed by 45 cycles of 95°C for 40 s, 60°C for 40 s and 72°C for 40 s on a Perkin-Elmer GeneAmp5700 Cycler. Primer design and data analysis were carried out as described above. Primer sequences are listed in Tab. 6.

Tab. 6 Oligonucleotid sequences for RealTime PCR

Splice Variante	*Sequence (5 → 3')*	*Primer*
TRPM8	5'-TATCTTACTGAACACCTGTAGTCCCAG-3'	Sense
	5'-TGAGTTTATAGTGTATTCAAAGCTGAGAAA-3'	antisense
16b	5'-CTGGAAGATTATCCTGTGTCTGTTTATT-3'	Sense
	5'-GTGCCTTGGTTTGTACCTAAATGA-3'	antisense
20b	5'-TTGGCTCAGATGGAAACAGCTT-3'	Sense
	5'-CGCGCGTGCTTCTGAAGT-3'	antisense
4a4b	5'-CATCGTGCTTATCAGGGAGAATG-3'	Sense
	5'-TCTGGGTGCCTTCCATGTG -3'	antisense
6b	5'-TGCTAGGTCACATGGAAGAAAGAC-3'	Sense
	5'-TGGCTGGCTCCATCACAGA-3'	antisense
Gegenstrang	5'-CACGAAGCTCTCCTGGGTTATT -3'	Sense
	5'-CCTTGTTGGAAATGGATCAGACA-3'	antisense
Avant13	5'-TCAGGTTTTCTTAATTGCAGAGCTT -3'	Sense
	5'- GGAGCATGCAGCTATTTGTTTG-3'	antisense
Avant25	5'-CCATTGTTTTTCAACTCTCTTTT-3'	Sense
	5'-GAAAGAGCCTTGTATTTGTTAAT-3'	antisense
beta actin	5'-TGCATTGTTACAGGAAGTCCCTT-3'	Sense
	5'-GGGAGAGGACTGGGCCAT-3'	antisense
NKX3-1	5'-ATTACTTGGTTTGTGAATCCATCTTG -3'	Sense
	5'-AGCTCTTCAGATGTTTTTCTACCAGTT -3'	antisense
MYC	5'-CGTCTCCACACATCAGCACAA- 3'	Sense
	5'-TCTTGGCAGCAGGATAGTCCTT -3'	antisense
KLK3	5'-GGCACTGGGAAGCCTAGAGA -3'	Sense
	5'-CCATCCCATGCCAAAGGA -3'	antisense

Relative expression analysis for Real Time PCR experiments was carried out using ΔΔCt-Method according to the manufacturer (Applied Biosystems). The Ct-value of beta actin and all other genes were measured at a threshold of 0.1. For normalization the Ct-value of beta actin was subtracted from the Ct -value of

the gene of interest. This ΔCt- value is the normalized expression value for each gene in each sample. In order to define the expression ratio between normal and tumor samples (ΔΔCt) the ΔCt-value of the tumor sample was subtracted from the ΔCt-value from the matched normal. Finally, the ΔΔCt was taken as the exponent of 2 in order to obtain the relative expression between the normal and the tumor sample. Statistic results were analyzed by 2-tailed Student's t-test. A $p< 0.05$ was accepted as the level of significance.

5.4 NORTHERN BLOT AND DOT BLOT ANALYSIS

Multiple Tissue Northern Blots and Matched Tumor/Normal Expression arrays were obtained from Clontech (Heidelberg, Germany). Hybridizations with α-^{32}P labeled DNA probe were performed according to the manufacturer's recommendations. Briefly, filters were prehybridized in ExpressHyb solution (Clontech, Heidelberg, Germany) for 30 min at 65°C. Gene specific probes were labeled with α-^{32}P dCTP by random hexamer priming and hybridized over night at 65°C with agitation. The filters were washed twice in 2x SSC, 1%SDS for 5 min and twice for 30min at 65°C and once at 0.1x SSC and if signal were too strong again for 30 min in 0.5% SDS at 65°C. Filters were exposed to a FUJI imaging plate and scanned in a FUJIfilm BAS-1800 II scanner. The 5' TRPM8 specific probe was obtained by digesting the TRPM8-pcDNA3.1/His clone (described above) with BamH1, which resulted in a 2713 bp fragment covering exons 1-20 from TRPM8. The 16b specific probe was derived by PCR using the primer pair (5'-ATTTAGGTACAAACCAAGGCACA-3' / 5'-AATTTCCAGGCTTTTTAATCATTT-3'). The 3' "unspecific" probe for TRPM8 was derived from the clone AI420227 which covers the TRPM8 sequence from base pairs 5258-5641 cloned into pT7T3D-Pac. This construct was digested with NOT1 and EcoR1 receiving the 384 bp probe.

5.5 IN SITU HYBRIDIZATION

The tissues used for hybridization were obtained from the Department of Urology at the University Hospital Charité and from a commercial distributor (Ambion, Huntington, UK). Sections were deparaffinized and rehydrated before fixation in 4% paraformaldehyde. After washing twice with PBS tissues were digested with proteinase K and hybridized over night with the sense and antisense probe at 65°C. The TRPM8 specific probe (and the control [sense] probe) was synthesized by in vitro transcription from a linearized TRPM8-pcDNA3.1/His plasmid (exons 1-20) and the RNA was transcribed from the T7 site using digoxigenin-labeled riboprobes according to the manufacturer's directions (Roche Applied Siences, Mannheim, Germany). After 3 h of transcription the probe was analyzed for purity and size on a 1% denaturing agarose gel. Hybridized probes were detected by using the alkaline phosphatase conjugate anti-DIG antibodies and signals were visualized using the NBT/BCIP

substrates. The sections were counterstained, mounted, and examined by a pathologist.

5.6 CELL CULTURE

The cell lines were obtained either from ATCC (American Type Culture Collection, Manassas, VA, USA) or the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells were routinely maintained according to the manufacturer's conditions, supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C and 5% CO_2. All cells were cultured free of antibiotics.

5.6.1 Culture of LNCaP cells for androgen activation

The human prostate cancer cell line LNCaP was obtained from ATCC and grown on RPMI 1640 medium supplemented with 10% charcoal-stripped fetal bovine serum (CCS) (HyClone, Utha,USA) at 37°C and 5% CO_2. The cells were plated in 6-well culture (Primaria, BD Biosciences, Heidelberg, Germany). For androgen induction LNCaP cells were grown for 24h in serum free RPMI 1640 to deplete undesired steroids for 24h prior experiment. Cells were then treated with 5% CCS FBS and the non-metabolizable synthetic androgen R1881 (Dupont-NEN, Life Science Products Inc., Boston, MA, USA) was added at concentrations of 1 µM, 10 µM and 100 µM. Equivalent amount of solvent (ETOH) was added to control cells. Cells were treated for 24h and harvested in GTC buffer and stored at -80°C before mRNA preparation.

5.7 PLASMID CONSTRUCTION

5.7.1 TRPM8

The TRPM8 ORF was cloned into the pcDNA3.1-V5-His-TOPO (Invitrogen, Karlsruhe, Germany) for transfection experiments in HEK293 cells by PCR using the primer pair 5'-GGCCGCCATGTCCTTTCGGGCAGCCAGGCT-3' and 5'-AAAGAGATTGCTAATAAAATCAAA-3'. Reaction was performed using the Elongase Amplification system (Invitrogen, Karlsruhe, Germany), at the following conditions: 95°C for 2 min, followed by 32 cycles of 95°C for 30 s, 62°C for 30 s and 68°C for 6 min. Subsequently PCR products were digested with the appropriate digestion enzyme, purified and cloned into BAMHI / XBAI restriction sites of the pcDNA6-myc-his vector (Invitrogen, Karlsruhe, Germany). For *in situ* hybridization experiments the TRPM8 gene was cloned into the pcDNA3.1 and digested with BAMHI, resulting in a 2.7 kb probe starting from the 5`end of TRPM8 gene.

5.7.2 16b

16b was cloned into the pcDNA6-myc-his vector (Invitrogen, Karlsruhe, Germany) 16b was amplified from a prostate adenocarcinoma Marathon-Ready™ cDNA (BD Biosciences, Heidelberg, Germany) by PCR using the primer pair 5'-CATGTTTACGGCTCTCATAAAGGA-3' / 5'-AGGATCTAGATCATGAGAGCACACCATATGGTG-3' (3' BSTXI and XbaI restriction site at the 3' end). Reaction was performed using the Elongase Amplification system (Invitrogen, Karlsruhe, Germany), at the following conditions: 95°C for 2 min, followed by 32 cycles of 95°C for 30 s, 62°C for 30 s and 68°C for 3 min. Subsequently PCR products were digested with the appropriate digestion enzyme, purified and cloned into BAMHI / XBAI restriction sites of the pcDNA6-myc-his vector (Invitrogen, Karlsruhe, Germany).

5.7.3 Promoter constructs

The 1.9 kb DNA fragment, which contains the 5' upstream region of TRPM8 was amplified by PCR using the primer pair 5'-TCTACGCGTGCCTGGCCCAATACTGCAT-'3 / 5'-TTCCTCGAGGCTTGACAATAACACCATAGTATGAAATC-'3 from the BAC AC005538, which was used as a template (restriction sites *MLUI* and *XHOI* for directional cloning are underlined). The 200 bp construct was derived also by PCR using the primers 5'-TCTACGCGTCCCATTTCATGAGGATGCTTACT-'3 / 5'-TTCCTCGAGACCCTAAGTGACGGTTTTTGTCAA-3' from the same template. Both fragments were digested with the restriction enzymes *MLUI* and *XHOI*, purified and isolated by elution from an agarose gel electrophoresis. The fragment was ligated into the *MLUI* and *XHOI* of the luciferase reporter vector pGL3-Basic vector (Promega, Wisconsin, USA), that had been digested previously with *MLUI* and *XHOI.*

5.8 LUCIFERASE REPORTER ASSAY

LNCaP, PC3, DU-145 and HEK293 cells were cultured as recommended by the supplier. For luciferase reporter assay cells were grown to 80% confluence in 96-well plates and in transiently transfected with 0.2 µg of the appropriate (200bp-pGL3, 2kb-pGL3, 2kb-pGL3-SD, pGL3-empty) reporter constructs. The phRL-null vector (Promega, Madison, Wi, USA) was used for transfection normalization. Each promoter-construct was at least transfected in quadruplicates. Transfections were carried out according to the manufacturer's description. Briefly, the DNA was mixed with 25 µl transfection medium (Opti-MEM, Invitrogen) and incubated for 20 min with 0.5 µl of lipofectamine diluted in 25 µl of Opti-MEM. The mixture was then applied to the cells in a total volume of 100 µl according to the manufacturer's directions. After 24 hours of incubation, cells were washed in PBS and lysed with 25 µl of Passive Lysis Buffer (Promega, Madison, Wi, USA). Promoter activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wi, USA)

according to the manufacturer's recommendations by using the Luminometer Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany). In transfection experiments for androgen induction cells were grown on RPMI 1640 medium supplemented with 10% charcoal-stripped fetal bovine serum (CCS) (HyClone, Utha, USA). Treatment of R1881 and transfection of cells were carried out in parallel for 24h. All other conditions remained the same. The median of all experiments normalized to transfection efficiency was calculated and standard deviations were calculated.

5.8.1 Site-directed mutations

Site-directed mutations of the 1.9kb-TRPM8-pGL3 construct were done following the instructions of the manufacturer using the primer pairs indicated in Fig. 50. The schematic view of altered transcription factor binding sites is described in (Fig. 49).

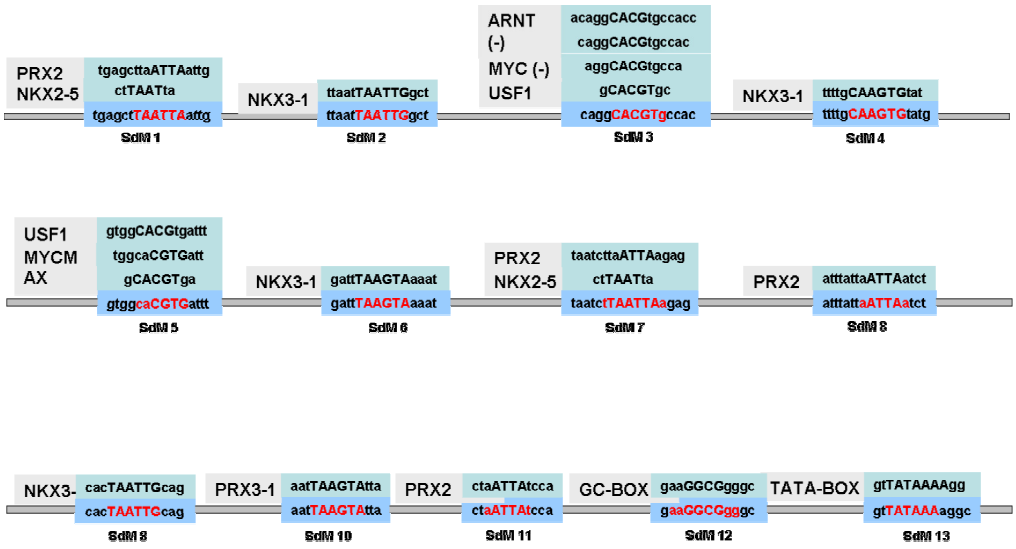

Fig. 49 Schematic view of site-directed-mutations of the 1.9-TRPM8-pGL3 vector. Red letter in blue boxes indicate the base pairs deleted in Site-directed-mutations (SdM)-pGL3-construct. Light green boxes show the sequence of each transcription factor binding site. Capital letters demonstrate the core binding nucleotides of each transcription factor binding site.

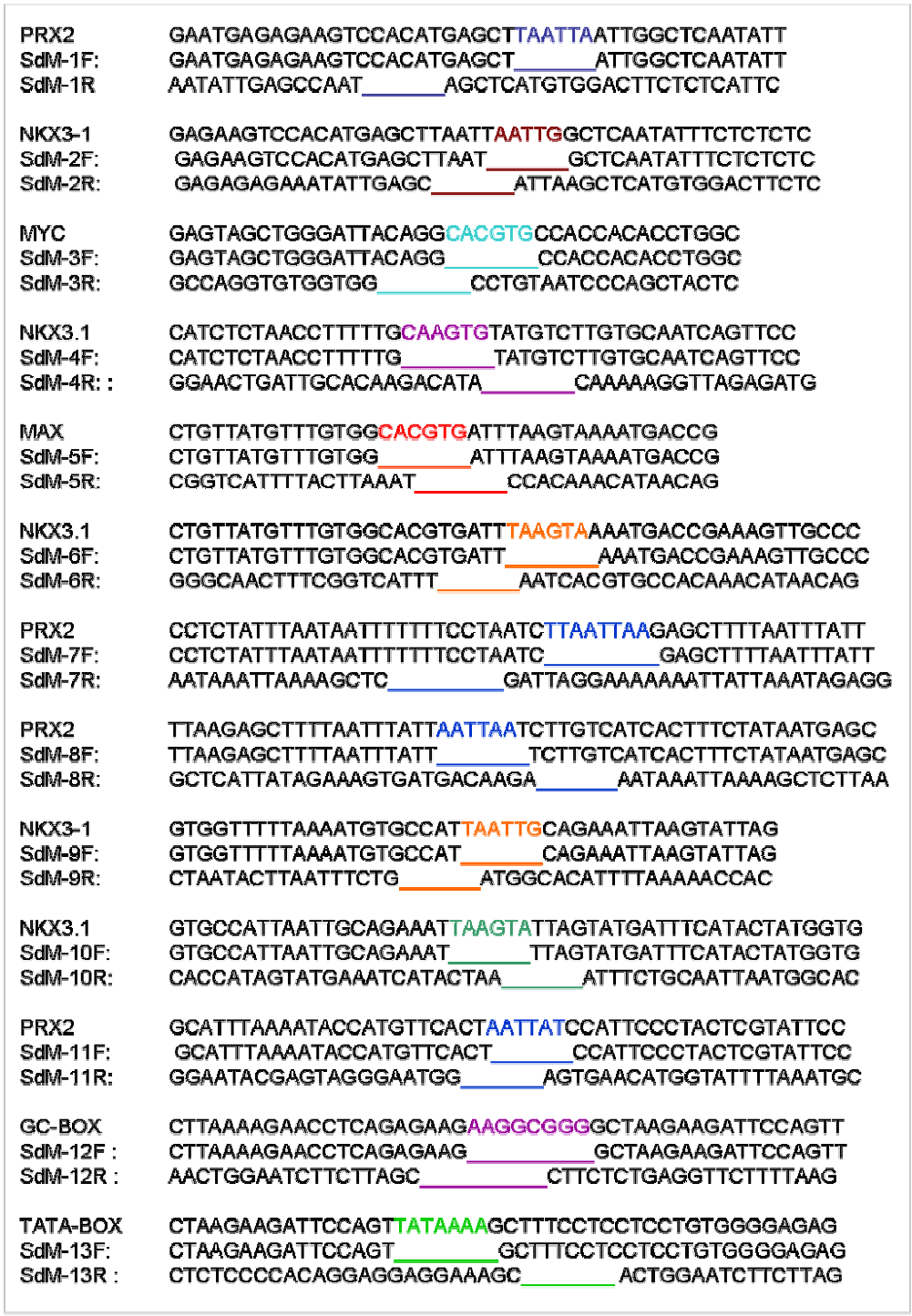

Fig. 50 Primer for the TRPM8 promoter site-directed mutations. Red letters indicate deleted base-pairs of the 1.9kb-TRPM8-pGL3 contruct.

5.9 INTRACELLULAR CA^{2+} MEASUREMENTS

5.9.1 Fura-2 assay

Changes of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were monitored by measuring fura-2 fluorescence. Cells were incubated with culture medium containing 1 µM fura-2-AM for 45 min. Thereafter, cells were washed with the extracellular bath solution described above. Fluorescence measurements in single cells were performed at room temperature with a digital imaging system (T.I.L.L. Photonics, München, Germany). Fura-2 fluorescence was excited at 340 nm and 380 nm wavelength and changes in $[Ca^{2+}]_i$ were monitored based on the ratio of the fluorescences obtained. The concentration of free $[Ca^{2+}]_i$ was calculated according to [Grynkiewicz, 85]. For calibration of free $[Ca^{2+}]_i$, fluorescence in the presence of EGTA (1 mM) or ionomycin (1 µM) was obtained. If drugs were added from dimethyl sulfoxide (DMSO) containing stock solutions, the solvent concentration did not exceed 0.1% which did not change Fura-2 fluorescence in control experiments. To avoid influence of DMSO on $[Ca^{2+}]_i$, we used DMSO in the bath solution at a concentration lower than 0.1%.

5.9.2 FLIPR assay

The Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, München, Germany) provides a rapid, high-throughput screening system for reading intracellular fluorescence assays such as $[Ca^{2+}]_i$ flux and membrane potential. The effects of drugs, agonist or antagonist on calcium channels or cell surface receptors like GPCRs can be quantified in cell based assays with the FLIPR system.

The Calcium Flex Station Kit offers an easy and fast fluorescence-based assay for detecting changes in intracellular calcium without washing steps. Assays were performed according to protocol of supplier. In brief, cells were seeded in 96 black well clear bottom plates and grown to 100% confluency at the day of measurement. Medium was changed before assay started. 100 µl medium per well were supplemented with 50 µl of freshly prepared calcium-assay reagent including the calcium specific fluorochrome and incubated for 30 min at 37°C. Another 96 well plate containing all stimulating reagents was prepared and placed next to the cells plate in the FLIPR device. All substances were tested in dilutions series at least in sextuplets. The pore forming agent ionomycin (1-10 µM) and/or ATP (10 µM) were used to compare shape, status and calcium flux of different clones (positive control for high $[Ca^{2+}]_i$ influx signal). HBSS buffer without substances were as negative controls and DMSO or ethanol in appropriate dilutions were used as solvent controls. After the baseline detection for 30 sec the FLIPR pipettor added 50 µl of substances to each well. Alterations of fluorescence signal which indicates calcium influx was detected with a laser at 488 nm. Signals from all 96 wells were monitored in parallel for up to 5 min with points from every 1-10 sec. Results were calculated using the FLIPR software.

5.10 FLOW CYTOMETRY ANALYSIS

For flow Cytometry assays, HEK293 cells were stable transfected for TRPM8-pcDNA3.1-V5-his and 16b-pcDNA6-myc-his were grown on 6 well plates. Cells were washed once with PBS and centrifuged at 300 g in PBS for 5 min. Cell pellets were resuspended in washbuffer (WB) (PBS with 0.2% BSA and 0.1% sodium azide) and centrifuged again at the same conditions. Cells were fixed with 300 µl CellFIX (BD Biosciences, Heidelberg, Germany) and incubated for 10 min, washed with PBS and centrifuged at 300g for 5 min. Cells were permeabilized with 300 µl Permeabilizing Solution 2 (BD Biosciences, Heidelberg, Germany) for 10 min and washing procedure was repeated as above. Staining was done with primary anti-V5 antibody (rabbit) (Invitrogen, Karsruhe, Germany) and anti-myc (mouse) (Invitrogen, Karsruhe, Germany) antibody for TRPM8 and 16b, respectively. The antibodies were diluted 1:100 in WB and cells were incubated for 10 min at RT. Cells were washed once in WB and centrifuged. Labelling was done with secondary fluorescence labeled antibody in 60 µl antibody solution at a concentration of 1:300 for 10 min in the dark. Anti-rabbit-phycoerithrin (PE) labeled and anti-mouse Fuoresceine Isothiocyanate (FITC) labeled antibody (Dianova, Hamburg, Germany) were used for V5 (TRPM8) and myc (16b) detection, respectively. The wash step was repeated and the cells were resuspended in 300 µl WB. Then cells were then analyzed by a FACSCalibur (BD Biosciences, Heidelberg, Germany) with the CELLQUEST program (BD Bioscience).

5.11 FISH ANALYSIS

These experiments were performed at the Charité, AG Tumorgenetik und Molekulare Zytogenetik at the Institut für Medizinische Genetik of Prof. Dr. Evelin Schröck.

5.11.1 Metaphase preparation

For preparation of metaphase chromosomes LNCaP cells were cultured in 6 well dishes for 72 h at standard conditions. Colcemid (KaryoMAX Colcemid Solution, Invitrogen, Karlsruhe, Germany) at a concentration of 10 µg/ml was added and cells were cultured for an hour at 37°C followed by standard metaphase preparation. Cells were harvested and centrifuged at 1200 g for 12 min. 0.4% potassium chloride was added to the cell pellet and incubated for 10 min. After hypotonic incubation, cell suspensions were washed several times in fresh fixative solution (methanol/acetic acid fixative vol/vol 3:1). Fixed cells were dropped onto clean slides at a volume of 20 µl and air dried for approximately 1 min. Before storage at -80°C slides were washed in increasing ethanol concentrations and dried for two days at 37°C.

5.11.2 Probe preparation and hybridization

The BAC (Bacterial artificial chromosome) clone AC005538 which was used as a probe for hybridization, maps to 2q37.2 covering the complete genomic region of TRPM8 plus an additional 100 kb of the 5' site of TRPM8 gene. 2 µg of DNA was used for Nick-translation using biotinylated-dUTP at 15°C for 1.5 hours. The DNA was precipitated and added to the hybridization buffer. Before hybridization of the slides were equilibrated in 2 x SCC at RT and treated with RNase and pepsin. Hybridization was performed as described elsewhere in detail [Lichter, 95]. Briefly, hybridization was performed overnight with the biotinylated AC005538 probe and the LSI C-MYC (8q24.12-q24.13) probe which was directly labeled with SpectrumOrange (1:200; Vysis Inc., Downers Grove; IL, USA). Staining was done with an avidin-FITC labeled antibody (Dianova, Hamburg, Germany) at a concentration of 1:200 for 45 min at 37°C. After repeated washings, slides were dehydrated, air dried and antifade treated.

Images were acquired through a Leica DM RXA epifluorescence microscope (Leica Microsystems, Solms, Germany) which was connected to a CCD camera). Image processing and data analysis was performed with QFISH software (Leica Microsystems).

5.12 FRET ANALYSIS

Fluorescence resonance engergy transfer analysis (FRET) was performed at the Freie Universität Berlin, Institut für Pharmakologie by Michael Schäfer and Daniel Sinnecker. FRET is used to measure the close proximity of fluorescence molecules (distance closer than about 12 nm), as for example the assembly of ion channels subunits in living cells. The method is describe in detail in [Schaefer, 02; Amiri, 03]. Briefly, TRPM8 and 16b were C-terminaly fused to CFP (pcDNA3-YFP) and YFP (pcDNA3-CFP) (Invitrogen, Karlsruhe, Germany). The absence of mutations was confirmed by DNA sequencing. HEK293 cells were transiently cotransfected (either pcDNA3-TRPM8-YFP and pcDNA3-TRPM8-CFP, pcDNA3-TRPM8-YFP and pcDNA3-16b-CFP [and vice versa] or pcDNA3-16b-YFP and pcDNA3-16b-CFP [vice versa]) for 16 hours using the Fugene 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) following the maufacturer's protocol. All experiments were performed in living cells using HEPES-buffered saline, containing 138 mM NaCl, 6 mM KCl, 1mM$CaCl_2$, 1mM $MgCl_2$, 5,5 mM glucose, 10 mM HEPES (pH 7,4), and 0.2% (w/v) bovine serum albumine. Cells were grown on coverslips and mounted in custom-made chambers. An inverted microscope (Axivert 100, Carl-Zeiss, Göttingen, Germany) connected to a monochromatic light source was used for digital fluorescence videoimaging (Polychrome II, TILL-Photonics, Martinsried, Germany). For fluorometric data analysis, regions of interest were defined over single cells. Cells exhibiting large intracellular aggregates of fluorescence were excluded from the analysis. A dual reflectivity dichroic mirrow (Chroma, Brattleboro, VT, USA) in combination with the Plan-Apochomat 63x/1.4 objective (Carl Zeiss) was used to excite fluorescent CFP at

415 nm and YFP at 515 nm. Fluorescence emission was filtered using a Lambda 10/2 motorized filter wheel (Sutter Instruments, Novato, CA) and recorded with a 12-bit CCD camera (IMAGO, TILL-Photonics). Emitted light of CFP was measured through a 460-500 nm emission band and YFP trough 535 -580 nm band pass filter.

The acceptor bleach protocol consisted of 30 cycles with 10 - 20 ms of exposure to detect the CFP and YFP fluorescence without YFP bleach and 80 -120 additional cycles with an additional 2 s of illumination at 510 nm to bleach YFP.

5.13 SEQUENCING

All cloning products were verified by sequencing using either gene specific primers or vector specific primers following the method developed by Sanger [Sanger, 77]. Approximately 500 ng of plasmid DNA were used for each sequencing PCR using the BigDyeTM Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Weiterstadt) at the following conditions: 95°C for 4min; 30 cycles of 95°C for 30 s, 50°C for 10 s; 4 60°C 4 min. Samples were purified from unincorporated dNTPs through Sephadex-G50 columns (Amersham Biosciences, Freiburg, Germany), dried, denatured and mixed with 3 µl of formamide loading buffer. Fragments were separated on a 5.25% polyacrylamid gel (PAGE plus, Ameresco, Solon, USA) in a ABI 377A DNA Sequencer (Applied Biosystems, Weiterstadt). Sequence assembling, editing and alignment was done using the GAP4.4-program [Bonfield, 95].

5.14 CONSTRUCTION OF STABLE CELL LINES

Stable transfections of the appropriate contructs were carried out using the Lipofectamine 2000 system (Invitrogen, Karlsruhe, Germany) according to the manufacturer's description. Briefly, HEK293 cells were seeded in dishes of 6 cm diameter and vector-DNA was mixed with 250 µl transfection medium (Opti-MEM, Invitrogen) and incubated for 20 min with 10 µl of Lipofectamine 2000 diluted in 250 µl of Opti-MEM. The mixture was then applied to the cells in a total volume of 2 ml according to the manufacturer's directions. About 16 h post transfection, medium was changed and supplemented with 5 µg/ml blasticidin (pcDNA6-myc-his or 1mg/ml neomycin (pcDNA3.1-V5-his-TOPO). At this point of time cells were transferred to a 96-well dish, plating approximately 1 cell per well. After 10-20 days cell growth was checked and wells with single clones were further incubated in 24-well dishes and analysed in Western Blot experiments for expression of constructs.

5.15 WESTERN IMMUNOBLOTTING

Protein samples were washed twice with ice-cold PBS and lysed with ice-cold NP-40 buffer (25 mM Tris-HCl, pH 7.6;1% NP-40 (IPEGAL); 150 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM Na_3VO_4;1 mM Pefabloc SC (Biomol,

Hamburg, Germany) and protease inhibitor-cocktail Complete (Roche, Lörrach, Germany). Cells were passed 3 times through a pipette and centrifuged at 14,000 x g for 10 min at 4°C. Protein content was measured using Bradford (Bio-Rad Laboratries, München, Germany). 12 µg of protein from cell lysates were denatured and in sample buffer, subjected to 3-8% Tris-Acetate gel (Novex/Invitrogen, Karsruhe, Germany), and thereafter transferred onto a nitrocellulose membrane. The blot was blocked in 5% milkpowder/TBST (0.05%) for an hour and probed with anti-myc-HRP antibody 1:5000 (Invitrogen). Signals were visualized using the enhanced luminescence ECL detection system (Amersham, Pharmacia Biotech, NY, USA).

5.16 IMMUNOSTAINING

Cells for immunostaining were grown on 24-well glass cover slips (12 mm, (Roth, Karsruhe, Germany) and washed twice with PBS. Fixation was done in 300 µl of (-20°C) methanol/ acetone (ratio 1:1) at RT for 5 min. Cells were washed 2x 5 min with PBS and permeabilized with 0.2% Triton-X100/PBS for 5 min. Blocking of cells was done in 0.2% fish gelantine (Sigma, Mannheim, Germany) for an hour. Staining for SV 16b was done with anti-myc (mouse) and anti-V5 (rabbit) at a 1:100 dilution in fish gelantine/PBS for one hour at RT. Secondary labeling was done at the same conditions with fluorescence labeled antibodies at a concentration 1:300 for 20 min in the dark. Alexa 594 donkey anti-Rabbit IgG and Alexa 488 donkey anti-Mouse IgG (both Molecular Probes, Leiden, Netherlands) were used for V5 (TRPM8) and myc (SV 16b) detection, respectively. Cells were washed once in 0.2% fish gelantine/PBS and nuclei (chromosomes) were stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma) for 5 min at RT. Dishes were mounted with Mowiol containing 0.1% DABCO (Sigma) onto glass slides.

Analysis was done on an inverted Laserscanning-Mikroscope Leica TCS SL (Leica, Solms, Germany); Laser: Ar 50mW 488nm, HeNe 1mW 543nm, HeNe 10mW 633nm. The application software Leica LCS (Version 2.585), LCS Multi-color software, objective PL APO 63x / 1.32-0.60 Oil was used for imaging analysis.

6 References

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Attachment

Sequences of Splice Variants

Explanations to attachments: letters underlined indicate sequences distinct from TRPM8. “Normal” letters are identical to the TRPM8 sequence.

SEQ ID NO: 1 (6b)

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SEQ ID NO: 6 (avant25)

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Data of Prostate cancer Patient

Tab. 7 Prostate Cancer Patient histopathological and follow up data of samples hybridized to the metg001 Cancer-Chip. R = 1 → Relapse after S; S = Surgery (radical prostatectomy); R = x → patient dead; GG = Gleason Grading,

Patient Number	TNM staging	Gleason Grading	Gleason_sum	Pre-surgery PSA	PSA-Relapse	month after S- Relapse free)	GG_microdiss	Age
Pr25	4	5+4	9	14,5	x	x	4	51
Pr8	3b	3+5	8	10,5	1	9	3	71
Pr41	2b	4+3	7	6,4	0	36	4	64
Pr45	3a	3+4	7	18,4	x	x	4	68
Pr111	3a	4+3	7	17,0	0	31	4	67
Pr82	2b	3+4	7	12,0	0	30	3	52
Pr94	3a	3+4	7	n.a.	0	39	3	60
Pr127	2b	3+5	8	5,8	0	31	2	51
Pr108	3a	2+2	4	20,5	0	34	2	54
Pr79	2b	3+3	6	12,0	0	30	3	69
Pr47	2b	2+3	5	8,4	0	36	3	65
Pr61	3a	3+5	8	n.a.	x	x	2	68
Pr70	3a	3+4	7	8,9	0	30	4	59
Pr65	2b	2+5	7	16,6	0	32	3	61
Pr85	2a	2+3	5	n.a.	0	19	3	60
Pr2	3a	4+3	7	14,0	x	x	3	61
Pr48	3a	2+3	5	30,4	0	29	2	60
Pr19	2b	2+2	4	4,0	0	33	2	59
Pr21	3b	4+3	7	3,3	0	27	5	78
Pr117	3a	4+4	8	18,3	0	18	3	67
Pr116	2b	2+2	4	19,0	0	9	1	58
Pr34	3a	3+4	7	n.a.	0	42	3	72
Pr125	2b	4+3	7	11,2	1	34	4	60
Pr3	2b	2+3	5	3,1	0	38	3	53
Pr102	2b	2+4	6	11,9	0	23	3	59
Pr49	3a	3+4	7	10,7	1	26	4	47
Pr28	3a	3+5	8	n.a.	x	x	3	66
Pr72	3a	3+4	7	15,0	0	37	4	66
Pr78	3a	5+3	8	10,4	0	2	3	58
Pr77	3a	3+3	6	15,0	0	33	4	61
Pr103	2b	3+4	7	n.a.	0	43	3	59
Pr88	3b	3+4	7	n.a.	1	43	3	66
Pr23	2b	3+4	7	40,6	0	10	3	67
Pr73	3a	3+5	8	16,3	1	12	3	60
Pr89	2b	2+3	5	n.a.	0	42	3	62
Pr29	2	2+3	5	5,6	0	32	3	69
Pr46	2b	3+2	5	25,3	0	32	3	62
Pr76	2b	2+4	6	9,3	1	13	3	70
Pr75	3a	2+3	5	4,9	0	28	3	54
Pr24	4	5+4	9	14,5	x	x	3	51
Pr96	3b	4+5	9	12,6	0	15	3	60
Pr17	3a	3+5	8	6,0	0	29	3	66
Pr118	2b	2+3	5	n.a.	0	34	3	56
Pr123	3a	3+4	7	n.a.	0	38	4	58
Pr14	2b	2+3	5	8,3	0	24	3	65
Pr204	2	2+5	7	10,4	1	32	3	67
Pr40	4	3+4	7	n.a.	1	13	3	65
Pr60	2b	4+3	7	13,1	0	11	3	46
Pr68	2b	2+3	6	n.a.	0	10	3	65
Pr69	3b	4+3	7	n.a.	0	38	5	68
Pr80	2b	1+2	3	4,1	0	37	3	63
Pr81	3a	2+5	7	n.a.	0	10	1	58

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit von mir selbst angefertigt wurde und keine anderen als die angegebenen Hilfsmittel verwendet wurden.

Weiterhin erkläre ich hiermit, dass von mir weder diese noch andere Dissertationen zum Promotionsverfahren an einer anderen Fakultät als der Humboldt-Universität zu Berlin eingereicht wurden.

Berlin, den 30.12.2003

Simone Kaiser